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Healthy Aging

Effects of bioactive molecules and natural extracts with antioxidant action in the modulation of aging mechanisms

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Summary

The aging population grows every year, and certain nations have the largest percentage of elderly people. An aging population necessitates a growth in health care services since they are more likely to have chronic age-related disorders, which leads to unsustainable health care expenses. Taken as a whole, the notion of "Healthy Aging" has been advocated for research to enhance the lives of the elderly. Several prior research on various nutraceuticals have been conducted to assess their potential as supplements to promote healthy aging and may even postpone the onset and progression of some age-related disorders. As a result, the antioxidant and anti-inflammatory effects of several nutraceuticals were investigated in various tissues (myoblasts, retinae, and brain).

- Project 1: Muscles provide a variety of important functions, including glucose absorption, bone density regulation, and protein homeostasis. Exercise is critical for maintaining muscle function, avoiding hypercontraction injury, and delaying the onset of age-related muscular diseases. There is a large change in magnesium and potassium ions during exercise, which, if unstable, can induce muscular damage. The purpose of Project 1 was to evaluate the ability of a combination containing magnesium, potassium, vitamin D3, and curcumin (MKVC) to prevent or restore muscle damage on C2C12 murine myoblasts under normal conditions and in the presence of caffeine to mimic a hypercontractility condition by studying cell viability, mitochondrial activity during exercise, calcium and magnesium movements during the muscle contraction/relaxation cycle, the metabolic changes, alterations in the various intracellular pathways. All the data gained will aid in the development of a formulation that will assist athletes in maintaining peak performance with protection against muscle injury and decreasing muscle loss due to aging as well.
- Project 2: As glaucoma is the leading cause of permanent blindness, defined by structural changes in the optical nerve, various nutraceuticals have demonstrated their ability to lower the oxidative stress associated with age-related eye illnesses. As a result, in Project 2, a hypothesis concerning a combination of gastrodin and vitamin D3 together with vitamin C, blackcurrant, and lycopene has been proposed to combat the early modifications of glaucoma

that lead to degeneration. RGC cells and retinal tissues were employed to test our theory under normal or H_2O_2/NMDA-induced glaucoma circumstances by assessing cell survival, ROS generation, identifying changes in the retinal area, and examining the various intracellular pathways. All of these will contribute to the development of a formulation that will aid in the slowing of the many deteriorations associated with glaucoma.

- Project 3: Concerning the many deteriorations affecting the brain as a result of aberrant protein depositions or a decrease in BDNF expression associated with multiple age-related neurodegenerative disorders and cognitive function loss. Because BDNF has a reduced ability to cross the blood-brain barrier, it was proposed that using a low-dose BDNF sequentially kinetic activated (SKA) formula could overcome this problem. This hypothesis was tested through BDNF quantification in both in vitro and animal models, as well as studying the safety of using this formula through cell viability, ROS production, mitochondrial activity, and determining the different intracellular pathways. Based on these findings, a combination of curcumin, vitamin D, and 6-shogaol (CVS) might delay and repair the damage caused by either oxidative stress or iron damage as two distinct characteristics of brain aging, and neurodegeneration were investigated. Another important point is to verify if CVS might promote BDNF expression in astrocytes and if it can aid with neuroprotection when administered to SH-SY5Y cells. These assumptions were tested by measuring cell survival and ROS generation, as well as identifying BDNF synthesis and distribution, and screening the several active intracellular pathways. All of this will pave the way for the development of a novel human anti-aging solution forneuroprotection.

Scopo della tesi

L'invecchiamento della popolazione cresce ogni anno e alcune nazioni hanno la percentuale più alta di anziani. Una popolazione che invecchia necessita di una crescita dei servizi sanitari poiché è più probabile che abbia disturbi cronici legati all'età, il che porta a spese sanitarie insostenibili. Nel complesso, il concetto di "invecchiamento sano" è stato sostenuto per migliorare la vita degli anziani.

Sono state condotte diverse ricerche su vari nutraceutici per valutare il loro potenziale uso come integratori per promuovere un invecchiamento sano posticipando l'insorgenza e la progressione di alcuni disturbi legati all'età. Di conseguenza, sono stati studiati gli effetti di diversi nutraceutici in vari tessuti (quali mioblasti, retina e cervello).

- Progetto 1: I muscoli svolgono una varietà di funzioni importanti, tra cui l'assorbimento del glucosio, la regolazione della densità ossea e l'omeostasi delle proteine. L'esercizio è fondamentale per mantenere la funzione muscolare, evitare lesioni da ipercontrazione e ritardare l'insorgenza di malattie muscolari legate all'età. C'è un grande cambiamento negli ioni magnesio e potassio durante l'esercizio, che, se instabile, può indurre danni muscolari. Lo scopo del Progetto 1 era valutare la capacità di una combinazione contenente magnesio, potassio, vitamina D3 e curcumina (MKVC) nel prevenire o ripristinare il danno muscolare sui mioblasti murini C2C12 in condizioni normali e in presenza di caffeina per imitare una condizione di ipercontrattilità, studiando la vitalità cellulare, l'attività mitocondriale durante l'esercizio, i movimenti di calcio e magnesio durante il ciclo di contrazione/rilassamento muscolare, i cambiamenti metabolici, le alterazioni delle varie vie intracellulari. Tutti i dati acquisiti aiuteranno nello sviluppo di una formulazione che aiuterà gli atleti a mantenere le massime prestazioni con la protezione contro le lesioni muscolari e riducendo anche la perdita muscolare dovuta all'invecchiamento.
- Progetto 2: poiché il glaucoma è la principale causa di cecità permanente, definita da cambiamenti strutturali nel nervo ottico, vari nutraceutici hanno dimostrato la loro capacità di ridurre lo stress ossidativo associato alle malattie degli occhi legate all'età. Di conseguenza, nel Progetto 2, è stata proposta un'ipotesi riguardante una combinazione di gastrodina e vitamina D3 insieme a vitamina C, ribes nero e

licopene per combattere le prime modificazioni del glaucoma che portano alla degenerazione. Le cellule RGC e i tessuti retinici sono stati impiegati in circostanze di glaucoma indotto da H_2O_2/NMDA valutando la sopravvivenza cellulare, la generazione di ROS, identificando i cambiamenti nell'area retinica ed esaminando i vari percorsi intracellulari. Tutto ciò contribuirà allo sviluppo di una formulazione che aiuterà a rallentare i numerosi deterioramenti associati al glaucoma.

- Progetto 3: Poiché il BDNF ha una ridotta capacità di attraversare la barriera ematoencefalica, è stato proposto che l'utilizzo di una formula SKA (Sequentialally kinetic activate) di BDNF a basso dosaggio potrebbe superare questo problema. Questa ipotesi è stata testata attraverso la quantificazione del BDNF in modelli sia in vitro che animali, oltre a studiare la sicurezza dell'utilizzo di questa formula attraverso la vitalità cellulare, la produzione di ROS, l'attività mitocondriale e la determinazione delle diverse vie intracellulari. Sulla base di questi risultati, una combinazione di curcumina, vitamina D e 6-shogaol (CVS) potrebbe ritardare e riparare il danno causato dallo stress ossidativo o dal danno del ferro poiché sono state studiate due caratteristiche distinte dell'invecchiamento cerebrale e della neurodegenerazione. Un altro punto importante è verificare se il CVS potrebbe promuovere l'espressione di BDNF negli astrociti e se può aiutare con la neuroprotezione quando somministrato alle cellule SH-SY5Y. Queste ipotesi sono state testate misurando la sopravvivenza cellulare e la generazione di ROS, oltre a identificare la sintesi e la distribuzione del BDNF e lo screening dei diversi percorsi intracellulari attivi. Tutto ciò aprirà la strada allo sviluppo di una nuova soluzione umana per la neuroprotezione.

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1. Introduction

Oxidative stress (OS) is defined as an impaired balance between the production of reactive oxygen species (ROS) and antioxidant defenses. Excessive imbalance on the side of the ROS generation leads to a disorder of the redox signaling that, if remains unsolved, triggers severe defects in cellular components that ultimately lead to cellular aging and various age-related diseases [1]. Several factors play a crucial role in the onset and progression of aging, such as environmental factors or telomere shortening; nevertheless, oxidative stress remains the main culprit in this phase of life [2]. According to a report by the World Health Organization (WHO), by 2030, 1 in 6 people worldwide will be 60 or older, reaching 1.4 billion, and this number will double by 2050. Moreover, the number of people who are 80 or older is expected to triple between 2020 and 2050, reaching 426 million [3]. It is known that biological aging is a normal, inevitable, complex, multifactorial, accumulative decline that affects molecular and cellular activities; this process occurs at multiple levels in biological systems causing a chronological gradual decline in physiological and functional capacities and can increase the risk of disease leading to death [4]. This condition is influenced by many factors, including lifestyle, environmental conditions, and genetic predisposition [5] and is characterized by genomic instability, epigenetic alterations, deregulated nutrient sensing, mitochondrial dysfunction, and altered intercellular communication [6]. However, oxidative stress remains a major factor in aging. Certainly, oxidative stress persists after an imbalance between the pro-oxidants and the antioxidant mechanisms. [7] This causes oxidative damage to various cellular components, and isn't only triggered by ROS production, but also by reactive nitrogen species (RNS) [8]. However, when an imbalance between removal and production of ROS occurs in favor of the latter, highly atomic unstable free radicals collide with a biomolecule and withdraw an electron by oxidizing it; for example, polyunsaturated fatty acids (PUFAs) through oxidative stress which cause an irreversible impairment of membrane fluidity and elasticity up to an eventual cell rupture [9; 10]. In this context, the end products of lipid peroxidation such as MDA, 4-HNE and F2-isoprostanes could accumulate in biological systems during oxidative stress and, due to the accumulation of ROS, the DNA bases can undergo oxidation, with consequent mutation and deletion. This process can occur in both nuclear and mitochondrial DNA, as seen in the production of 8-hydroxy-2-deoxyguanosine [11]. It is known that age-dependent decay is related to structural changes in the mitochondria, accompanied by alterations in the biophysical properties of the membrane that lead to changes in the electron transport chain complexes,

decreased membrane fluidity and consequent energy imbalance and mitochondrial insufficiency [12]. Therefore, the maintenance of healthy mitochondria is essential for maintaining cellular homeostasis as compromised mitochondria have been described as a common hallmark of numerous human diseases and aging [13].

New studies have established the correlation between ROS production, inflammation, and aging progression [14]. There is an association between asymptomatic chronic low-grade inflammation, resulting from chronic activation of the innate immune system, associated with ROS overproduction, and the onset and progression of aging, or age-related diseases such as cancer, atherosclerosis, and osteoarthritis [15]. The efficiency of the immune system decreases with advancing age. The adaptive immune system declines and the innate immune system becomes more active, which is reflected in an increase in the number of natural killer cells and an increased production of pro-inflammatory cytokines [16]. During the aging process, there are also increasing indications that the deregulation of the innate immune system leads to an increased production of inflammatory cytokines (TNF-α, IL-6 and others) which drive a low-grade chronic inflammatory state, which is known as inflammatory aging (referred to as inflamm-aging) [17; 18]. In this context, several studies have shown that the serum levels of proinflammatory mediators in the elderly (> 50 years) are 2 to 4 times higher than in younger people [19]. However, inflamm-aging also affects non-immune cells, including adipocytes, muscle, endothelial cells, and senescent cells [20-22]. Consequently, components derived from normal metabolism or dead cell debris can lead to inflammation when produced in large quantities [23]. These products include lipofuscins, advanced glycation end products (AGEs), tau protein aggregates, alpha-synuclein fibrils, and beta-amyloid (β-amyloid) networks recognized by receptors such as toll-like receptors (TLR), NOD-like receptors, AGE receptor (RAGE), and others collectively referred to as pattern recognition receptors (PRRs) [24-26]. These toxic end-products are not always easily neutralized or removed and can eventually build up; this accumulation results from a progressive impairment of the disposal mechanisms (proteasomes,autophagy, mitophagy, and chaperone-mediated autophagy) [27-292].

1.1 ROS and oxidative stress as the major causes of aging

ROS, commonly referred to superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$), consist of both radical and non-radical oxygen species resulting from the partial reduction of oxygen [30]. Cellular ROS are endogenously produced by the mitochondrial oxidative phosphorylation, cytochrome P450, endoplasmic reticulum, peroxisomes, and lysosomes [31]; or from exogenous sources under the influence of ultraviolet light, ionizing radiation, and xenobiotics [32]. Cellular ROS levels are tightly regulated by our evolved antioxidant machinery, which include both enzymatic and non-enzymatic antioxidants such as glutathione, uric acid, melatonin, vitamins C and E, and polyphenols [32; 33]. However, the first line of enzymatic antioxidants includes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). SOD catalyzes the reaction in which superoxide dismutase anions are converted to hydrogen peroxide, which can be further reduced to water by CAT or GPX [34; 35]. Since mitochondria are considered a major source of ROS, initial studies have shown that mitochondria release H_2O_2 under physiologic hypoxic conditions to activate the transcription factor hypoxia-inducible factor 1 (HIF-1), necessary for metabolic adaptation to low oxygen levels [36]. Subsequently, H_2O_2 released from mitochondria can activate c-Jun N-terminal kinase 1 (JNK1), p53, and nuclear factor-kappa B (NF-κB) [37]. All in all, this suggests that ROS released by mitochondria evolved as a means of communication between mitochondrial function and cellular processes to maintain cellular homeostasis and promote stress adaptation.

Much of the ROS produced are rapidly quenched by antioxidants [38; 39], but, when ROS production exceeds the threshold of cells to reduce their reactivity, cells begin to experience an oxidative stress state [40], in which lipids, proteins, and DNA are damaged. This damage may ultimately reduce cellular performance and contribute to senescence [41]. The main problem induced by ROS is related to their surplus production which leads to a phenomenon called oxidative stress, that can seriously damage cell membranes and other structures such as proteins, lipids, lipoproteins, and DNA [42; 43]. Lipoperoxidation of polyunsaturated fatty acids (PUFAs) is mediated by hydroxyl and peroxyl radicals that generate malondialdehyde (MDA), 4-hydroxy-2-non-enol (4-HNE) and F2-isoprostanes, which cause irreversible impairment of membrane fluidity and elasticity and eventually cell rupture [44]. In addition, ROS mediated irreversible protein oxidation by carbonylation, and tyrosine nitration causes structural changes and loss of enzymatic activity [45;46]. Finally, oxidative DNA damage implies the formation of different mutagenic oxidative DNA lesions which can cause mutations. However, the body has several mechanisms to counteract this oxidative damage,

either through the initial antioxidants defense, such as SOD, CAT, and GPX, which neutralize ROS before the generation of free radicals. If the damage persists, DNA repair systems, proteolytic enzymes that recognize, degrade, and remove damaged proteins, DNA, and lipids, play a role in preventing the damage from accumulating and rescuing the cells [47]. Accordingly, if not properly regulated, oxidative stress can contribute to the development and progression of several chronic and degenerative diseases, as well as aging and some acute pathologies (trauma, stroke etc.) through persistent damage of macromolecules. As in neurodegenerative diseases, a high mitochondrial ROS production, which depletes cellular antioxidants, reacts with nucleotides and proteins, triggering lipid peroxidation of membranes; consequently, cellular damage causes the release of damage-associated molecular patterns (DAMPs) to initiate an inflammatory response to eliminate damaged cells [48]. This inflammatory response also includes further ROS production from activated microglia, neutrophils, and macrophages that reach the site of injury [49]. Inflammatory cells release ROS at the site of inflammation, resulting in excessive oxidative stress [50], which can trigger intracellular signaling cascade of proinflammatory gene expression, such as NF-κB activation in response to inflammatory agonists [51]. Thus, based on these data, it can be confirmed that specific inflammatory agonists use ROS as part of their signaling cascades. In this context, Li et al. has demonstrated that NADPH oxidase (NOX)-2, which produces ROS, influences the assembly of an active interleukin-1 (IL-1) receptor complex in the endosomal compartment by controlling the H_2O_2-dependent binding of TRAF to the IL-1R1/MyD88 complex, that removal of both superoxide and H_2O_2 from within the endosomal compartment results in significant avoidance of IL-1β-dependent IKK and NF-κB activation [52]. Additionally, NOX4 and TNF-αactivity also plays a central role in amplifying the inflammatory response through increased ROS production; indeed, NOX4 is required for lipopolysaccharide-induced NF-κB activation [53], and TNF-α-induced NF-κB activation increases antioxidant expression, resulting in decreased TNFα-induced apoptotic signaling via ROS/JNK (c-Jun N-terminal kinase) pathway [54].

1.2. Pro-oxidant-Antioxidant Balance

Several degenerative diseases, such as atherosclerosis, and autoimmune diseases show an imbalance between pro-oxidants and antioxidants in favor of pro-oxidants [55]. **Pro-oxidants** are chemicals that induce oxidative stress by either generating ROS or by inhibiting antioxidant systems. They can be divided into several categories: drugs, redox-active metals, pesticides, physical activity, psychological anxiety, pathophysiological conditions, environmental factors, and even antioxidants [56]. Therefore, the body tries to restore cellular homeostasis through **antioxidants,** since any substance present in low concentrations compared to an oxidizable substrate will significantly delay or prevent the oxidation of that substrate [57]. The antioxidant activity is exerted in different ways: interruption of the spread of the chain reaction of free radicals, quenching of singlet oxygen, inhibition of the oxidation reaction of free radicals, inhibition of pro-oxidative enzymes, reduction of hyperoxides to stable compounds, chelators of metal prooxidants such as iron and copper [58-60]. Cellular redox homeostasis is carefully controlled by a sophisticated endogenous antioxidant defense system to normalize ROS levels, which includes endogenous antioxidant enzymes such as SOD, CAT, GPX, proteins and low molecular weight scavengers like uric acid, coenzyme Q, and lipoic acid [61]. Antioxidants are also obtained as part of the dietary supplement. Some dietary antioxidants cannot neutralize free radicals, but they can enhance the endogenous antioxidant activity. An ideal antioxidant should be easily absorbed and eliminate free radicals, chelate redox metals in physiologically acceptable body concentrations. They should also be able to act in both aqueous and membrane domains and positively influence gene expression [62].

Antioxidants are classified into:

- Endogenous antioxidants, which include enzymatic and non-enzymatic pathways. The primary antioxidant enzymes are SOD, CAT, and GPX. O_2 is converted to H_2O_2 by SOD, which is broken down into water and oxygen by CAT, preventing the production of hydroxyl radicals. In addition, GSH-Px converts peroxides and hydroxyl radicals into non-toxic forms oxidizing reduced glutathione (GSH) to glutathione disulfide (GSSG) and then reducing it to GSH by glutathione reductase. Other antioxidant enzymes are glutathione-S-transferase and glucose-6-phosphate dehydrogenase [63]. The non-enzymatic antioxidants are molecules that interact with reactive oxygen and nitrogen species (RONS) and stop the free radical chain reactions: such as bilirubin, vitamin E, and β-carotene are present in the blood,

while albumin and uric acid are 85% of the antioxidant capacity in plasma [64].

- Exogenous antioxidants that are ingested from food, such as ascorbic acid (vitamin C), scavenges hydroxyl and superoxide radical anion; α-tocopherol (vitamin E), protects against lipid peroxidation of the cell membrane; and phenolic antioxidants, which include stilbene derivatives (resveratrol, phenolic acids, and flavonoids), oil lectins, selenium, zinc, and drugs such as acetylcysteine [65]. Epidemiological studies have shown beneficial effects of regular consumption of fruit and vegetables rich in bioactive antioxidants and the prevalence of some degenerative diseases. These antioxidant compounds either reduce the further production of free radicals or neutralize those already formed and repair the damage caused by free radicals. Moreover, these natural antioxidants interact with endogenous antioxidants to increase their antioxidant capacity [66].

Pro-oxidant/antioxidant balance (PAB) is a state of dynamic equilibrium established in conditions of homeostasis between RONS produced and those scavenged by cells, body fluids, or other human body components. This equilibrium is subject to permanent dynamic changes, which are caused either by physiological (physical stress) or pathological factors (illness, presence of xenobiotics or UV radiation) [67]. Excessive ROS creates an imbalance of pro-oxidants and antioxidants, and the serum PAB can then rise [68; 69], but that rise is altered by antioxidants such as vitamins C and E and antioxidant enzymes [70; 71]. Various studies have shown that the cellular redox status is crucial for ROS-mediated signal transduction and the maintenance of mitochondrial function. [72] Certainly, intracellular GSH depletion remarkably promotes mitochondrial ROS production and eventually activates membrane depolarization [73], stimulating the Nrf2/ARE pathway which is a cardinal point for inducing antioxidant defense systems and modulating intracellular GSH in response to stress [74]. Keeping adequate intracellular ROS levels is of utmost importance in physiological redox signaling by activating and regulating endogenous defenses to protect cells from nitrosative, oxidative, and electrophilic stress [75] As reported by Limòn-Pacheco and his colleagues, when supplied with N-acetylcysteine, cells show an improvement in GSH depletion and restored ARE-associated transcriptional activity to baseline levels [76]. Antioxidants targeting the mitochondria show great potential to counteract ROS-dependent damage due to their abilities to cross the phospholipid bilayer of mitochondria and eliminate surplus ROS [77]. A broad range of antioxidants such as ubiquinol (MitoQ) target mitochondria via conjugation to the triphenylphosphonium cation [78]. In this regard, some popular antioxidants have been

reported to have pro-oxidative activity based on three factors: the presence of metal ions, the concentration of the antioxidant in the matrix, and the redox potential of the antioxidant [79-81]. For example, both vitamin C and alpha-tocopherol fall into this category, with vitamin C having an antioxidant effect at low doses (30-100 mg/kg body weight) and pro-oxidative effect in high doses (1000 mg/kg body weight) [82] and in the presence of iron and copper ions [83]; thereby these antioxidants could cause a slight oxidative stress due to their pro-oxidative properties [84]. Multiple evidence highlights that both the activities of antioxidant enzymes and the levels of low molecular weight antioxidants are altered as the organism ages [85; 86], and this has raised awareness of the possibility of using natural products with antioxidant activity to maintain this balance and to achieve a state of healthy aging [87].

The generation of ROS and the activity of the antioxidant defense appear more or less balanced *in vivo*. Indeed, this balance can be slightly tipped in favor of the ROS, so that there is a continuous ROS formation and accumulative little oxidative damage in the human body. This creates a need for a second category of endogenous antioxidant defense system, that removes or repairs damaged biomolecules before they accumulate and lead to altered cell metabolism and permanent damage, either by repairing oxidatively damaged nucleic acids, removing oxidized proteins by proteolytic systems, repairing oxidized lipids by phospholipases, peroxidases, and acyl transferases [88]. It appears that the failure of some or all the repair systems is more likely to contribute to aging and age-related diseases than moderate fluctuations in antioxidants and ROS formation [89-91].

Many of the essential maintenance and repair systems become deficient in senescent cells, frequently due to accumulated cell damage, for example, lysosomal accumulation of lipofuscin [92; 93]. Age-related oxidative changes are most pronounced in non-proliferating cells, such as neurons and cardiac muscle cells, as there is no “dilution” of damaged structures through cell division [94], as these cells lack the ability to activate the cyclin D-dependent kinase for cell cycle progression [95], that render them unable to repair DNA mutations or remove oxidized protein deposits, along with the decreased efficiency of endogenous antioxidant defenses, allowing damaged cells to remain and contribute to disease onset and progression. For this reason, increased fruit and vegetable consumption is associated with lower signs of cell damage *in vitro*, such as lower oxidative stress, DNA damage, malignant transformation rate. From an epidemiological point of view, they appear to lead to lower incidence of certain types of cancer and degenerative diseases such as ischemic heart disease and cataracts [96-98]. On the other hand, an increased or prolonged effect of free radicals can overwhelm the defense mechanisms against ROS and contribute to the development of diseases and aging. Since the

oxidative damage to our cells increases with age, the increased absorption of exogenous antioxidants from fruits and vegetables can support the body's own antioxidant defenses. Antioxidants such as vitamin C and E, carotenoids, and polyphenols (e.g., flavonoids), are currently considered to be the most important exogenous antioxidants. In summary, based on several studies that have correlated the different positive effects of natural active ingredients and vitamins either for protection or as therapeutic tools in neurodegenerative diseases, cancer,and autoimmune diseases, this has sparked the interest in introducing them as pharmaceutical products [99]

1.3. Nutraceuticals as possible way to healthy aging

The term nutraceutical has been proposed by Stephen De Felice (founder and chairman of the Foundation for innovation in Medicine (FIM)) as a combination between nutrition and pharmaceutical and defined it as *a food or a part of it that provides medical or health benefits including the prevention or treatment of a disease beyond the traditional nutrients they contain* [100; 101]. Nutraceuticals can be used to improve health, delay the aging process, prevent chronic diseases, increase life expectancy, or support the structure and function of the body [102].

The diets of the Western world are certainly richer than in the past. Therefore, advances in the food industry and people's poor knowledge of the basic principles of nutrition have led to a great use of unbalanced diets, high in calories and fat and low in protein, vitamins, and minerals. [103]. Through the advent of several degenerative diseases that have shown the importance of introducing nutritional supplements as a solution [104]. A dietary supplement is defined as a product that contains one or more of the following ingredients: minerals, vitamins, amino acids, herbal extracts, in the form of a concentrate, metabolite or ingredient, or a combination of these ingredients [105]. In this way, the body is not exhausted and at the same time injuries and fatigue are avoided.

Usually, nutraceuticals are contained in dietary supplements and have high antioxidant activity, which counteract the altered redox state during aging [106] or against various pathological conditions such as atherosclerosis [107], neurological diseases [108], cardiovascular diseases [109].

One of the main effects of nutraceuticals is their anti-inflammatory properties by inhibiting the activation of NF-κB, blocking the overexpression of tumor necrosis factor-alpha (TNF-α) and IL-1, downregulating the overexpression of cell adhesion molecules, inhibiting phospholipase

A2, cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), inducible nitric oxide synthase (iNOS), myeloperoxidase, inhibition of ROS-generating enzyme activity, increase of the ability to scavenge ROS and modulate intracellular signaling pathways [110-112].

Given that oxidative stress and inflammation are characteristic of unresolved oxidative stress and inflammation during normal aging and in several degenerative diseases and the resulting consequences and based on previous studies of the various effects of nutraceuticals as either antioxidants or anti-inflammatory agents.

For this reason, we have selected several herbal ingredients and vitamins to investigate the possibility of introducing them as a possible future supplement for protection or adjuvant therapy.

In this context the effects of some substances for nutraceutical use in 3 different areas of aging are analyzed: muscle, retinal and cerebral.

1.3.1 Nutraceuticals for muscle protection and recovery (project 1: muscle aging)

Skeletal muscle mass is a body tissue that plays an important role not only in movement and strength generation, but also in the metabolic regulation of the body. [113]. Muscle mass diminishes by up to 3–8 percent every decade starting at the age of 30–40 years, and the rate of loss accelerates to 15% per decade after the age of 70 [114]. A loss of muscle mass and strength is a reduced physical function that is observed in sarcopenia and is currently recognized as a clinical public health problem, especially in the aging population. [115; 116].

Although muscle mass loss usually occurs with age, the degree or rate of decline in muscle mass varies across the population, suggesting that several factors may affect muscle mass loss [117]. Muscle loss is believed to be faster in men than in women, mainly due to hormonal factors. [118] Lack of physical activity, malnutrition, chronic illness, insulin resistance, or mild metabolic acidosis have been linked to muscle wasting [119-122].

The consequences of sarcopenia are the gradual loss of an important metabolically active tissue, accompanied by a decrease in energy consumption and loss of physical function, which leads to frailty and disability. [123], Additionally, loss of bone density leads to an increased risk of fractures and increased insulin resistance and impaired blood glucose control, along with severe disturbances in electrolyte control [124].

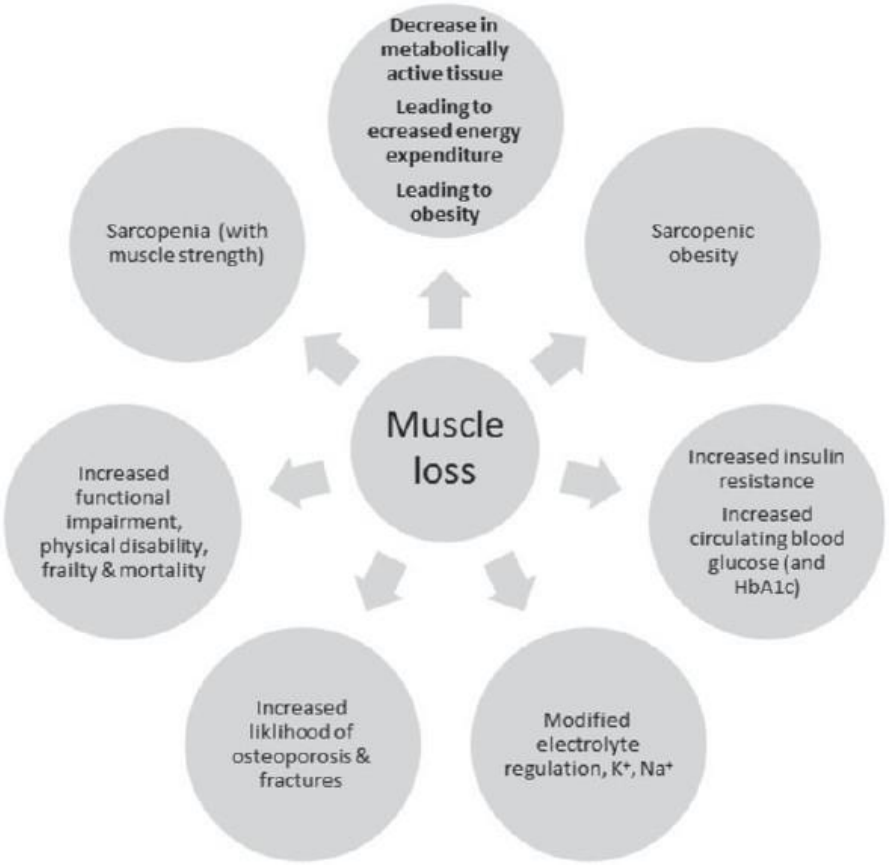

Figure 1: Metabolic, physiological, and functional consequences of losing skeletal muscle [125]

The fact that the primary focus of nutrition in the elderly is to focus on protein as a prevention and therapy option is beyond question. However, there are other aspects of nutrition that have been less well studied, although they may play an important role in preventing age-related muscle loss in middle-aged and older adults. [126-129].

Factors related to skeletal muscle loss:

Biological and lifestyle factors	Nutrients
Increased age Smoking habit Increased height and weight (> muscle) Reduced physical activity Reduced oestrogen and testosterone Cyclical weight loss	Protein, essential amino acids, leucineAlcohol Vitamin D Antioxidant nutrients (vitamin C, E, carotenoids) Minerals and trace elements (Se, Zn, K+, Mg2+, Fe, P) Dietary acid-base load (alkalinogenic diet) Dietary fat composition Bioactive compounds (nitrate, curcumin, and olive oil antioxidant compounds)

As muscle aging advances, a progressive age-related decline appears in the de novo synthesis of mixed muscle proteins, myosin heavy chains and mitochondrial proteins 130]; therefore, aged muscles are characterized by a defect in the ability of leucine to stimulate protein synthesis [131].

Four important signaling pathways regulate the size of myofibers and the contractile performance of the muscle crosstalk, modulate each other on different levels and at the same time coordinate protein synthesis and degradation simultaneously, namely IGF-1–Akt–FoxO pathway, myostatin, NFκB and glucocorticoids. In the IGF-1–Akt–FoxO pathway, Akt controls protein synthesis via mTOR and protein degradation via transcription factors of the FoxO family. FoxO also signals between protein breakdown and synthesis, with FoxO3 playing a role in suppressing protein synthesis and Akt playing a role in anabolism by suppressing protein breakdown [132].

Inflammatory cytokines (C-reactive protein, TNF-α) play a potential role in muscle mass loss through the action of inflammatory cytokines mediated by the NFκB transcription factors expressed in skeletal muscle [132; 133]. It has been found that high inflammatory cytokine status in the elderly 70–79 years of age at baseline was associated with loss of muscle mass and strength [134; 135].

Oxidative stress and the accumulation of ROS generated during oxidative metabolism, may contribute to age-related muscle loss [136]. In the elderly, oxidative stress in skeletal muscle can trigger the imbalance between protein synthesis and degradation, thus leading to greater cellular damage of DNA, proteins and membranes and associated functions of mitochondria as well. [137]. Oxidative stress can also lead to a loss of viability of satellite cells, resulting in a reduction in their lifespan and a decrease in their proliferative capacity, and can also affect

the structure of the myofibril and the functional state of calcium (Ca^{2+}) channels. responsible for muscle contraction [138].

The health benefits of regular physical exercise are beyond question. Both resting and contracting skeletal muscles produce ROS and RNS. Low physiological ROS levels are generated in muscles to maintain normal tone and contractility, but excessive ROS production promotes contractile dysfunction, which leads to muscle weakness and fatigue [139]. Perhaps this is why intense and prolonged exercise leads to oxidative damage to proteins and lipids in the contracting muscle fibers [140].

Regular exercise induces changes in both enzymatic and non-enzymatic antioxidants in skeletal muscle, which are essential for modulating multiple signaling pathways and regulating the expression of multiple genes in eukaryotic cells. The magnitude of exercise-mediated changes in skeletal muscle SOD activity increases with exercise intensity and duration [141; 142]. Mild physical activity increases NF-κB activity in the muscle of rats as well as the gene expression for manganese superoxide dismutase (MnSOD) and endothelial nitric oxide synthase (eNOS) [143].

When a muscle injury occurs, an inflammatory response is initiated to guide repair, although excessive inflammation can be harmful, in a way to dramatically reduce inflammation may not be the ideal solution for optimal recovery [144; 145]. For this reason, in recent decades both elite and amateur athletes increased the use of herbal products and dietary supplements capable of promoting muscle growth and reducing fat mass [146] both before and after physical activity. The function of these substances is to act as a free radical scavenger, to prevent or reduce oxidative stress, [147] to reduce muscle pain and physical stress, and to improve athletic performance [148].

1.3.1.1. Vitamin D3

Vitamin D has gained its initial fame for being a steroid hormone that acts through its specific receptors to affect target organs [149], it can be obtained either orally or by photolysis of its precursor within the skin [150; 151].

Vitamin D receptors (VDR) are expressed in a large number of human cell types, including skeletal muscle, suggesting a potentially widespread effect of vitamin D [152-154].

Regarding skeletal muscle metabolism, vitamin D increases calcium manipulation by increasing the activities of the calcium-binding protein (calbindin-D9K) in cellular sarcoplasm [155; 156], promoting muscle cell differentiation and proliferation, especially type 2 fibers, by acting on insulin growth factor I (IGF1) [157], while its non-genomic effect occurs by

activating a transduction signal that induces MAP kinase (MAPK) and phospholipase C (PLC) pathways, which in turn leads to a rapid Ca^{2+} influx [158; 159] as seen in figure 2.

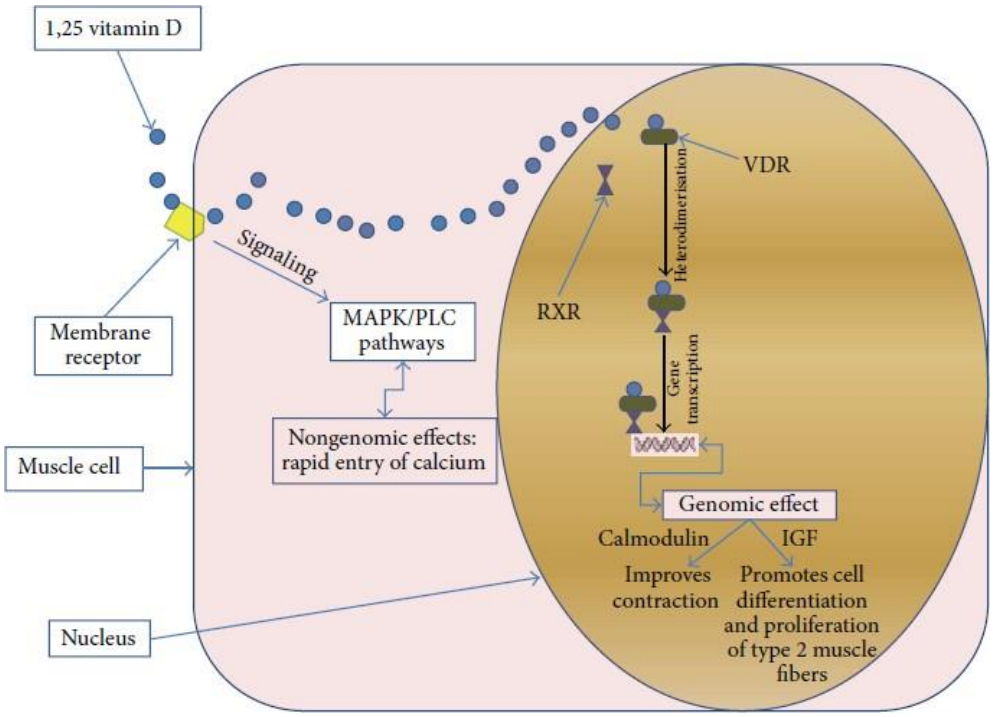

Figure 2: Effects of 1,25 $(OH)_2$ D3 on muscle cells [160]

Ceglia et al. has shown that in older women, vitamin D supplementation (4000 IU/day) for 4 months was associated with a 30% increase in intramyonuclear VDR concentration and a 10% increase in muscle fibers cross-sectional area, especially type 2 fibers [161]. The VDR exhibits several polymorphisms, some of which may have clinical significance, as Geusens et al. has shown that the presence (allele bb) or absence (allele BB) of a restriction fragment (BsmI) can determine muscle strength; that is, subjects with the bb phenotype had 23% greater muscle strength in the quadriceps than those with the BB phenotype [162].

Clinical studies on the elderly people have shown that low serum levels of 25-hydroxyvitamin D (25(OH)D) correlate with decreased muscle strength in lower-extremities and poorer performances as seen during standing up from a chair [163-166].

The Progetto Veneto Anziani (Pro.V.A.) has investigated the relationship between serum 25(OH)D levels and physical performance in older adults, including 2694 community dwelling elderly women and men in one community. The physical performance of the participants was assessed by means of tandem test, 5 time-controlled chair strands (TCS), 6-minute walking (6mW), handgrip strength and quadriceps strength. The results conclude that lower 25(OH)D values in women are associated with poorer coordination and weaker strength (TCS), slower

walking time and lower limb strength in men, and weaker aerobic capacity (6mW) in both genders. It was concluded that 100 nmol/L of 25(OH)D appears to be more beneficial for optimal physical performance in older men and women, and vitamin D supplementation should be encouraged to meet this threshold [167].

1.3.1.2. Curcumin

Exhausting physical activity, especially acute or high intensity with many eccentric contractions, leads to a state characterized by muscle damage and delayed onset muscle soreness (DOMS) [168]. In addition, exercise-induced muscle damage (EIMD) induces an inflammatory response associated with decreased muscle strength, decreased range of motion (ROM), localized swelling, increased muscle proteins in the blood like creatine kinase (CK) [169], along with an increase in other markers like C-reactive protein (CRP) and ILs-6, -8 and contributes to the production of ROS by promoting the activation of transcription factors such as NF-κB; thereby influences the performance of athletes [170-172].

Curcumin, also called diferuloylmethane, is a well-known spice used in curry in India and other Asian countries, is a natural polyphenol that has attracted attention for its anti-inflammatory, antioxidant, and anticancer activities [173]. Several studies have shown the tremendous beneficial effects of curcumin for better post-exercise recovery and prevention of muscle damage and performance improvement. A study was performed on 17 men who were instructed to take 5g of curcumin (2.5 g/dose, twice/day) 2 days before exercise to 3 days after exercise, it has reported that curcumin consumption 24-48h after eccentric single-leg press exercise reduced delayed onset muscle soreness (DOMS) during leg exercises, also having the supplement increases the athletic performance, suggesting that attenuated DOMS has a positive impact on performance and promotes recovery [174].

Tanabe and his colleagues conducted two experiments in which 20 men were asked to perform eccentric elbow exercises. In the first experiment, participants received curcumin supplementation of 180 mg (90 mg/dose) 7 days before exercise, while in the second experiment they received a supplementation 7 days after exercise. Results have shown no significant effect on DOMS in the pre-exercise curcumin supplementation group in contrast to the post-exercise curcumin supplementation group with significant DOMS reduction after 3-6 days [175]. The most likely mechanism by which curcumin supplementation can mitigate DOMS is by reducing the inflammatory responses that occur during the post-exercise recovery period. Curcumin inactivates NF-κB, which is an important mediator of inflammation, leading to a decrease in levels of other inflammatory mediators, including pro-inflammatory cytokine

mRNA and proteins [176]. In addition, curcumin can reduce the expression of the inflammation markers like intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM- 1) and attenuates the activity of 5-LOX and COX-2 [177].

Davis and his team have reported that curcumin supplementation significantly reduced levels of ILs-1β, -6, and TNF-α, which are cytokines that promote inflammatory responses after downhill running [178].

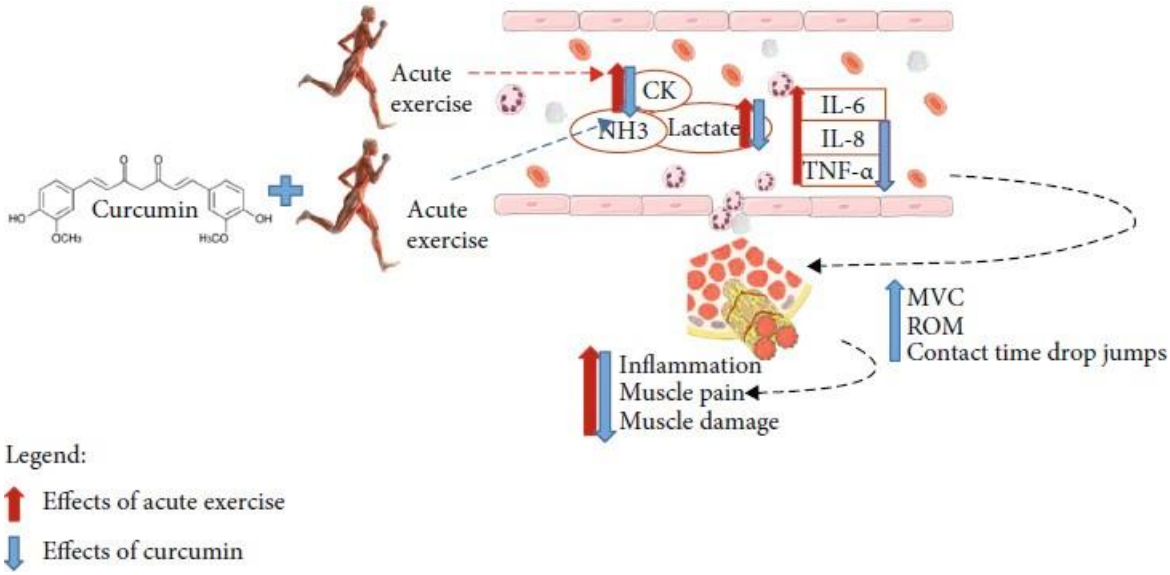

Figure 3: potential positive effects of curcumin supplementation during acute physical exercises [179]

Several studies have shown an association between curcumin supplementation and post-exercise inflammatory factors, in which McFarlin et al. **[**180**]** has reported a decrease in TNF-α and IL-8 levels after leg presses; the Tanabe' team also reported reduced IL-8 levels 12 hours after eccentric exercise [175]. In addition, Mallard et al. **[**181**]** has reported decreased thigh circumference after exercise and an increase in ILs-6, -10. Overall, curcumin supplementation, either before or after exercise, can protect against the effects of increased exercise-induced inflammation and increased ROS production associated with high energy demands, protect muscles from any deterioration, and relieve post-exercise pain.

For this reason, in **Project 1**, we aimed to investigate the effectiveness of a combination of magnesium, potassium as well as with vitamin D3, and curcumin on muscle cells in order to protect them under normal conditions or after physical exercise.

1.3.2. Nutraceuticals for Retinae protection (project 2: retinal aging)

Glaucoma, chronic optic neuropathy that affects more than 80 million people, is classified as the second leading cause of blindness worldwide [182], has been implicated in retinal ganglion cells (RGC) death [183], degenerative damage in the optic nerve together with damage to the trabecular meshwork (TM) to increase intraocular pressure (IOP) as a result of either increased production of aqueous humour or decreased outflow of aqueous humour [184] and subsequent loss of vision [185].

Glaucoma is categorized under age-related eye diseases with multifactorial etiology in which oxidative stress is considered to be the main responsible by inhibiting key enzymes of the tricarboxylic acid cycle, the mitochondrial electron transport chain, and the mitochondrial calcium homeostasis, which leads to a disturbed energy metabolism [186; 187]. Other factors are considered such as aging, genetic, epigenetic, and environmental factors [188; 189], neuroinflammation [190; 191], glutamate excitotoxicity due to overactivation of N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors [192].

Interestingly, several studies have found an association between dietary factors, nutraceuticals [193; 194] and the risk of glaucoma incidence, in which a deficiency in certain nutrients has been found in patients with glaucoma and supplementation can play a role in treatment [195]. Some nutraceuticals have shown ability to lower IOP [196; 197], increase the blood flow to the optic nerve [195], modulate the excitotoxicity and promote the survival of RGC [198-200].

Highlighting the key role of oxidative stress plays in RGC damage, antioxidant vitamins have been suggested as potential neuroprotective agents [201; 202], Yuki and his colleagues have found low vitamin C serum levels in Japanese patients with normal-tension glaucoma compared to normal controls [203]. Observational studies have determined an association between vitamin D and the risk of age-related macular degeneration (AMD), just as seen in a meta-analysis evaluation that has shown an association between low circulating levels of 25(OH)D (50 nmol/L (20 ng/ml)) with late-stage AMD, but this relationship wasn't observed in early-stage AMD [204]. On the contrary, several studies have found no association between serum vitamin levels and glaucoma prevalence in humans, such as the Nurses' Health Study and the Health Professional Follow-up study, which didn't find a strong association between the risk of primary open-angle glaucoma and vitamins C, A, and E consumption [205], a meta-

analytical study has found no association between different types of glaucoma and serum levels of vitamins B6, B12, or D3 [206].

1.3.2.1. Role of Vitamin D3 in the eye

It is now recognized that the role of vitamin D is not limited to bones but also targets a larger number of non-bone organs, including the eye [207]. Vitamin D3 has been shown to play an important role in reducing inflammation, modulating the immune response, and reducing angiogenesis in the eye, making vitamin D a protective factor for glaucoma, and its deficiency could explain the onset and severity of glaucoma in some patients [208]. Recent evidence suggests that serum vitamin D status may be a biological determinant related to visual function and retinal structure in older adults. With increasing age, a decreasing proportion of older adults can maintain normal vitamin D status (from 50% at the age of 60 to less than 20% after the age of 90) [209], vitamin D deficiency has been linked to decreased visual acuity in older adults [210], for a variety of reasons, including decreased macular health [211].

Recently, Kutuzova et al. has shown from the results of their studies in mice that $1,25(OH)_2D$ suppressed the expression of several genes related to the regulation of IOP. They also found that topically applied $1,25(OH)_2D$ reduced IOP in non-human primates. This could suggest that vitamin D also plays a role in regulating IOP in humans [212]. Recently, Goncalves and his colleagues have reported that vitamin D deficiency was linked to primary open-angle glaucoma (POAG) [213].

1.3.2.2. Gastrodin

Gastrodin is a natural phenol and one of the components of *Gastrodia elata BI* [214]. Gastrodin could cross the blood-brain barrier and rapidly break down to p-hydroxybenzyl alcohol (HBA) in the brain [215]. Recent studies have shown that gastrodin has a neuroprotective effect against hypoxia-induced toxicity in cultured cortical neurons [216], alleviated cerebral damage after transient focal cerebral ischemia [217], and protects primary cultured rat hippocampal neurons against Aβ peptide-induced neurotoxicity [218]. All these findings suggest that gastrodin may be effective in mitigating neuroinflammation that is implicated in various neurodegenerative diseases.

Since microglial cells are the only resident immune cells in the retina, they are readily activated in the events of trauma, inflammation, or immunological stimuli. The action of gastrodin has been tested on microglial activation and has exhibited its neuroprotective effects in a model of

acute ocular hypertension (AOH), in which it has inhibited AOH-induced microglia activation and the production of proinflammatory mediators (TNF-α and iNOS) and at the same time reduces the loss of RGCs in a dose-dependent manner. In addition, the level of phosphorylated p38 MAPK was significantly reduced by continuous gastrodin treatment [219].
Previous studies have reported that gastrodin inhibits the expression of iNOS, COX-2 and proinflammatory cytokines in cultured lipopolysaccharide (LPS)-stimulated microglia via MAPK pathways [220].

The effect of gastrodin and the underlying mechanism were examined in a model of high glucose (HG)-induced human retinal endothelial cell (HREC) injury, results have demonstrated that HG has induced HRECs apoptosis accompanied by increased ROS production, HG stimulation decreased the levels of SIRT1, which is accompanied by an increase in TLR4 expression and the level of phosphorylated NF-κBp65. Treatment with gastrodin has significantly reduced HG-induced apoptosis and oxidative stress, as well as reducing the activation of the (SIRT1)/TLR4/NF-κBp65 signaling pathway in HRECs exposedto HG [221].

1.3.3. Nutraceuticals for neuroprotection (project 3: brain aging)

The brain undergoes normal aging without clinical related neurodegenerative pathologies or deposits and is characterized by a significant decrease in the speed of information processing, problem solving, reaction time, speed of movement, hand and foot coordination, work, and long-term memory along with spatial orientation [222; 223]. However, the brain is an easy target for oxidative damage under the influence of high energy demands with high oxygen consumption, high levels of iron and polyunsaturated fatty acids, a sensitive blood supply, and little protection from the body's own antioxidant machinery [224]. Since mitochondria are the main source of ROS during mitochondrial respiration, which makes them the main center for oxidative stress; that when ROS production exceeds brain threshold it leads to redox imbalance, neurotoxicity, genomic instability, pro-inflammatory gene transcription, and cytokine release [225], along with damage and inactivation of parts of the electron transport chain through an ROS feedback cycle [226]. Age-related loss of mitochondrial integrity and functionality affects both expression and processing of the amyloid precursor protein (APP) for further production of amyloid-beta oligomers that are deposited into plaques, as observed in Alzheimer's disease (AD) [227], it was shown that amyloid-beta can produce oxidative stress as it forms complexes

with redox-active metals such as copper, zinc, and iron, which promote its aggregation into metal-amyloid complexes, which leads to excitotoxicity, promotion of membrane depolarization and impairment of mitochondrial function [228]

Normally, after the loss of neurons and the support of neuroglial cells, the brain gradually loses weight at a rate of about 0.1% neurons/year between 20-60 years and by the age of 90 the brain mass decreases by about 11% [229; 230], increased pigmentation is observed in aged nerve tissues due to the deposition of two pigments: a brown, lipofuscin is associated with the deposition of amyloid protein and the formation of neurofibrillary tangles [231], and a black pigment, neuromelanin [232], however, the density of these abnormal areas is often low during normal brain aging, but at high densities in neurodegenerative diseases [230]. The brain volume decreases, as can be seen after a neuronal loss in the cerebral cortex, the grooves that mark the surface convolutions are visibly deeper in the brains of older people in both the frontal and parietal lobes than in the temporal and occipital cortex, and these structural changes are also related to poor memory [233], the ventricles expand due to the progressive loss of the lining cells, followed by a ventricular enlargement filled with cerebrospinal fluid to fill the space left by the reduction in volume of the brain, the leptomeningeal complex dilates slightly as the subarachnoid space increases [234]. Genes involved in the synaptic function and plasticity, vesicular transport, mitochondrial functions, and calcium homeostasis show reduced expression after the age of 40; Antioxidant enzymes and growth factors, which are expected to inhibit the effects of oxidative damage, are altered due to changes in the effectiveness of their signaling pathways or their low production with aging [235].

Under normal physiological conditions, human brain cells consume 20% of the total oxygen intake compared to other organs [236]. Since mitochondria are a major source of ROS production, changes in organelle structure reduce detoxification capacity, resulting in imbalanced ROS levels [237].

Because of their high metabolic rate of myelin production and maintenance, oligodendrocytes are susceptible to oxidative damage [238] which requires high concentrations of ATP and iron [239].

Despite the high need for ROS detoxification, the brain contains significantly lower concentrations of antioxidants than other body organs, and oligodendrocytes contain low concentrations of GSH. Suboptimal antioxidant levels make it difficult to combat the large amounts of highly reactive polyunsaturated fatty acids, iron, and ROS, which accumulate with increasing age [240] particularly in the cerebral cortex, in the hippocampus, in the striatum, and in the hypothalamus [241; 242]. Post-mortem brain tissue analyses have shown a

significant decrease in the antioxidant activity of SOD, CAT and GST in the hippocampus and frontal cortex [243], similar reductions have been reported in connection with increased protein oxidation in the substantia nigra of aging individuals [244].

Age-related increases in ROS promote immunosenescence in the CNS by activating inflammasome complexes [245; 246], promoting cytokine maturation and pyroptosis and are strongly expressed in oligodendrocytes [247]. IL-1β is a specific target of inflammasomes involved in the development of AD pathology [248]. IL-Aβ can induce tau phosphorylation and Aβ neurotoxicity, which causes direct damage to the myelin sheaths [249], Aβ-interactions with neuronal membranes can also induce inflammasome activation when potassium efflux occurs in Aβ ion channels [250]. These pathological interactions result in a chronic low-grade inflammatory condition that decreases cellular antioxidant capacity and causes aggregate damage to macromolecules [251].

Among the various molecules and nutraceuticals known, BDNF, Vitamin D3, curcumin and ginger were selected, given the properties of greatest relevance for the thematic.

1.3.3.1. Brain-derived neurotrophic factor (BDNF)

BDNF, the most abundant neurotrophin in the mammalian CNS, is necessary for the survival of various neuronal populations during the development and formation of neuronal networks in the adult brain. It increases the activity of antioxidant enzymes and fights free radicals, inhibits the excitatory effects of amino acid cytotoxicity and apoptosis, exerts its biological effects through tyrosine receptor kinase B (TrkB), for ongoing neurogenesis and for various forms of synaptic plasticity [252-257].

BDNF promotes the formation and maturation of excitatory and inhibitory synapses [258], it potentiates excitatory synapses at pre- and post-synapses; BDNF presynaptically increases glutamate release [259; 260] while postsynaptically increasing the NMDA receptor [261; 262]. BDNF regulates the maturation of γ-aminobutyric acid (GABAergic) synapses in the hippocampus for the inhibitory synaptic transmission [263; 264], it upregulates the expression of voltage-gated Ca^{2+} and Na^{+} channels on the plasma membrane [265].

It has also been shown that BDNF can interact with oxygen radicals (ROS), the imbalance of ROS is implicated in the mechanisms of aging, neurodegenerative diseases, and some neuropsychiatric disorders [266]. Several clinical studies have shown changes in the blood concentration of BDNF in patients with neuropsychiatric disorders such as major depression

[267], and AD [268]. Studies carried out both *in vitro* and *in vivo* have shown that the expression of BDNF and its specific receptor TrkB are essential for obtaining adequate numbers of proliferating stem cells, for the differentiation of neuronal populations and for the maturation of excitatory synapses [269].

Changes in BDNF levels could contribute to the shrinkage of the hippocampus in late adulthood because it is highly concentrated in the hippocampus [270-272].

In humans, a single nucleotide polymorphism in the BDNF gene influences its regulated secretion in the hippocampus [273] and has been associated with its lower serum levels [274] and smaller hippocampal volumes [275-277], is also present in the caudate nucleus, but less concentrated than in the hippocampus [278-281]. Therefore, serum BDNF levels would be related to decreases in hippocampal volume and memory performance. In rodents, BDNF moderates synaptic plasticity and neurogenesis in the dentate gyrus and is directly related to learning rates in spatial memory paradigms [282-284], by blocking either the release of BDNF or the binding of BDNF to its TrkB receptor, long-term potentiation is effectively eliminated in the hippocampus [285]. In addition, induction of BDNF production and secretion in the hippocampus can save long-term potentiation and alleviate spatial memory deficits in aged mice [286; 287]. BDNF also moderates tau formation [288], blocks β-amyloid neurotoxicity [289], and the hippocampus-dependent memory performance in animal models of AD [290; 291]. Post-mortem studies have found reduced BDNF levels in the hippocampus of older adults compared to younger adults and lower levels in people with AD and PD compared to controls of the same age [292; 293].

However, there is a lack of therapeutic interventions that can slow down neurodegeneration and help repair and regenerate neurons in the affected brain [294]. To help patients with dementia or stroke recover quickly from these debilitating conditions, a potential drug should have the ability to promote neurite growth and synaptic plasticity through improved neurotrophic support by increasing levels of endogenous neurotrophins such as nerve Growth Factor (NGF), BDNF, glial cell-derived neurotrophic factor (GDNF), neurotrophin-3 (NT3), neurotrophin-4 (NT4), and/or enhancement of adult neurogenesis in the affected circuits [295].

Data have shown the importance of neurotrophins in the treatment and prevention of numerous age-related neurodegenerative diseases. Among them, BDNF is described as a modulator of normal brain aging, is a growth factor, the secretion of which depends on neural activity and its brain level decreases with age, is required for changing the density of the dendritic spines that underlie learning and memory disorders, who are disturbed during senescence [290]. Because BDNF deficiency is observed in AD patients, it has been suggested that the introduction of exogenous BDNF could be a potential therapeutic intervention for AD. Nonetheless, exogenous BDNF supplementation has several obstacles, the most important being the amount of BDNF from its exogenous supplement that could reach the affected neurons as BDNF can't cross the blood-brain barrier [296; 297]. If the amount is too low it may not be enough to achieve the desired effect, on the contrary, if it is too high it can be so dangerous that it causes a downregulation of the TrkB receptors. The second problem is the selectivity of BDNF action, as only certain areas of the brain suffer from decreased BDNF levels and that BDNF overload could cause several problems, such as increased neural excitability and aggravated seizure development [298].

Trying to resolve these problems, Xu et al. developed a rat model of AD to investigate the effect of exogenous BDNF on okadaic acid-induced cognitive impairment, in which the intrahippocampal injection of okadaic acid resulted in decreased spatial learning ability and reduced memory along with suppressed BDNF expression in the rat hippocampus. However, all of these impairments were attenuated by increasing the expression of BDNF, TrkB, and synaptophysin (SYN) proteins, along with reducing tau phosphorylation by enhancing the activity of phospholipase A2 (PP2A) after intrahippocampal of 50ng/ml human full-length BDNF protein injection. Using the neuroblastoma SH-SY5Y cell line in the same experiment, treated with okadaic acid for the AD model and treatment with exogenous BDNF, the results showed that BDNF activates PP2A and inhibits tau phosphorylation via the PI3K/GSK-3β pathway [299].

1.3.3.2. Curcumin

Curcumin is the most active element of turmeric (*Curcuma longa*), an herb of the ginger family that has been used in oriental medicine for millennia [300]. Turmeric is made up of three major polyphenol compounds called curcuminoids, curcumin, demethoxycurcumin (DMC), and

bisdemethoxycurcumin (BDMC); of these, curcumin has a low molecular weight and is the most abundant, making up about 77% of the curcuminoids [301], It has better antioxidant and anti-inflammatory effects compared to the other two curcuminoids [302]. Curcumin consists of two aryl rings that contain ortho-methoxy phenolic hydroxyl (OH) groups that are symmetrically bonded to a β-diketone moiety. The occurrence of intramolecular hydrogen atom transfer on the β-diketone chain of curcumin leads to the existence of keto- and enol-tautomeric conformations in equilibrium and this contributes to its powerful antioxidant and anti-inflammatory properties [303]. The o-methoxy group that flanks the molecular structure of the curcumin base makes it a powerful free radical scavenger, the hydrogen bond interaction between the phenolic OH and the o-methoxy groups strongly modulates the OH bond energy so that the hydrogen atom can be acquired by free radicals to stabilize them [304]. Coupled with the molecular structure of curcumin that makes it a powerful antioxidant, its ability to bind to KEAP1 due to its electrophilic properties makes it a strong NRF2 inducer. Curcumin acts as an electrophile and binds to KEAP1 via the sulfhydryl (SH) group, leading to cysteine modification and stabilizing NRF2 [305]. Stabilization and separation of NRF2 from KEAP1 results in its nuclear translocation, and transcription from ARE induces phase II antioxidant genes that activate enzymes synthesizing GSH [304; 306].

However, with age, the well-controlled system of the brain gradually loses its integrity and leads to continued transcription of NF-κB, inducing systemic chronic low-grade inflammation. Some of the downstream NF-κB-induced proteins, TNF-α, ILs-1β and -6, are potent NF-κB activators and form an auto-activating loop [307;308].

In human studies, it has been reported that circulating levels of proinflammatory cytokines increase with age; Elevated levels of TNF-α, IL-6 and -1β have been reported in both the peripheral and central nervous system (CNS), leading to age-associated, chronic, low-grade systemic inflammation, also known as inflammaging [309; 310].

The β-diketone moiety in the molecular structure of curcumin is a Michael's reaction acceptor, which by binding to IKKβ inhibits the nuclear translocation of NF-κB [302; 311; 312].

Belviranli and colleagues gavaged 20-month-old female Wistar rats with 300 mg/kg curcuminfor 12 days and behavioral tests were performed after 7 days of curcumin treatment. Curcumin supplementation has improved spatial memory and decreased MDA levels in the brain [313]. In another study, three doses of curcumin (100, 200, and 400 mg/kg) were tested in normally aging, middle-aged (12 months) rats over a period of 6 months. To analyze the effects of aging, serum was collected to measure albumin, globulin, and albumin/globulin ratio; they also measured CRP, lipid peroxidation products, total antioxidant capacity, SOD,

and nitric oxide (NO). Rats fed the two high doses had significantly lower CRP levels compared to the sham control and low dose treated groups. However, rats fed the highest dose also had high levels of MDA, suggesting that curcumin can induce lipid peroxidation at higher doses. Rats given the two high doses also had high levels of NO in their plasma. The results from this study show that the beneficial effects of curcumin are dose dependent and higher doses of curcumin may not necessarily be beneficial as they show a hormetic effect [314].

In addition, results from studies using a normal aging model show that even without severe pathological conditions, curcumin can slow age-related cognitive decline, as well as oxidative stress and inflammation, two processes that have been linked to the progression of cognitive impairment [314].

Curcumin dose-dependently inhibited the formation of beta amyloid fibrils (fAbeta) from Abeta (1–40) and Abeta (1–42) and their elongation dependently destabilizing preformed fAbetas. The effective concentrations (EC_{50}) of curcumin for the formation, extension, and destabilization of fAbetas were in the order of 0.1–1 μM [315]. Abeta (1–40) or fAbeta (1–42) activates the early growth response-1 (Egr-1), a nuclear transcription factor, which leads to increased expression of cytokines (TNF-α and IL-1β) and chemokines (MIP-1β, MCP-1, and IL-8) in monocytes, Curcumin (12.5–25 μM) suppressed the activation of Egr-1-DNA-binding activity and abrogated Abeta (1–40)-induced expression of these cytokines and chemokines. Curcumin exerts this activity by inhibiting Abeta (1–40)-induced MAP kinase activation and the phosphorylation of ERK-1/2 and its downstream target Elk-1. Curcumin also inhibits the Abeta (1–40)-induced expression of CCR5 and chemoattractant-induced chemotaxis of THP-1 monocytes [316].

1.3.3.3. The role of Vitamin D3 in the brain

The regional and cellular localization of the VDR is remarkably similar in the human and rat brain; both neurons and glia have been shown to express the VDR [317]. The intense expression of the VDR in large cells in the substantia nigra, which contains the highest concentration of dopaminergic (DA) neurons in the brain, is highly relevant, as $1,25(OH)_2D3$ has been shown to increase the expression of tyrosine hydroxylase in adrenal medullary cells *in vitro* [318]. The involvement of vitamin D in central nervous system function is supported by the presence of the enzyme 25(OH)D3-1*a*-hydroxylase, which is responsible for the formation of the active

form of vitamin D, as well as the presence of VDRs in the brain, mainly in the hypothalamus and in the dopaminergic neurons of the substantia nigra, whereby vitamin D becomes a neurosteroid [317], this suggests both autocrine and paracrine effects for calcitriol on nerve cells [319].

The influence of the active form of vitamin D on the nervous system has been linked to changes in the production and release of neurotrophic factors such as NGF for neuron differentiation, as well as an increase in GDNF levels. In addition, vitamin D has been shown to significantly increase the rate of neurite outgrowth when added to hippocampal explants [319]. Moreover, 1,25 $(OH)_2$D3 is an important factor that modifies the synthesis of neuromediators such as acetylcholine *via* increased gene expression of the enzyme choline acetyltransferase (CAT) [320; 321]. Vitamin D has also been found to affect the expression of genes associated with GABA-ergic neurotransmission [322], and to stimulate the expression of tyrosine hydroxylase (TH), which is responsible for catecholamine biosynthesis [318; 323]. The neuroprotection of vitamin D involves the synthesis of proteins that bind Ca^{2+} ions (e.g., parvoalbumin) and thus maintain cellular calcium homeostasis, which is very important for the function of brain cells [324-326]. In addition, it was shown that the administration of 1,25$(OH)_2$D3 down-regulates L-type voltage-sensitive Ca^{2+} channel expression in rat hippocampal cultures, and this indicates the protective effects of the hormonal form of vitamin D on the brain by reducing Ca^{2+} influx into the neurons [327]. Based on studies on immature rats, vitamin D has also been shown to modulate the opening of the L-type calcium channel opening via non-genomic effects through various kinase pathways and enzyme activities in the cerebral cortex [328]. Studies on rat neuron culture have shown that 1,25$(OH)_2$D3 increased glutathione levels because GSH delivered to nerve cells by astrocytes is a fundamental antioxidant that protects cells from ROS-induced oxidation and resulting apoptosis. This suggests an important neuroprotective effect of vitamin D3 by counteracting oxidative damage to the central nervous system [329; 330]. In addition, vitamin D inhibits the synthesis of iNOS to prevent a cascade of neurotoxicity and neuron death, since nitric oxide is a precursor of peroxynitrite NO^-_3, which in turn leads to the deactivation of several enzymes by reacting, for example, with sulfhydryl (-SH) groups, as well as through damage to mitochondria and disruption of cellular energy processes [331; 332].

1.3.3.4. Ginger

Ginger, the rhizome of Zingiber officinale Roscoe (Zingiberaceae family), is a widely used food ingredient and is widely prescribed in traditional medicine for curing various symptoms such as the common cold, nausea, asthma, cough, bleeding, and muscle pain [333; 334], was also combined with other prescription drugs for brain disorders, such as paralysis from ischemic stroke and a nerve relaxant [335].

Chemically isolated components from ginger are divided into pungent and flavoring compounds; pungent constituents from ginger include gingerols, shogaols, zingerones, gingerdiols, gingerdione, and capsaicin [336]. 6-Shogaol, a dehydrated form of 6-gingerol, is another important pungent ingredient in dried ginger [337], is used as a marker compound for quality control of ginger extracts, commercial products, and raw materials [338]. Recent studies have shown that 6-shogaol is more stable than 6-gingerol with stronger pharmacological effects due to the thermal liability by the presence of β-hydroxyl keto group [339; 340].

Many studies have shown that 6-shogaol has potent anti-AD activity, improves memory, and strengthens the antioxidant system [341; 342], inhibits inflammatory mediators and improves cognitive function in both A" (1–42) and scopolamine-induced dementia mouse models by increasing the NGF levels and postsynaptic proteins in the hippocampus [341]. In relation to H_2O_2 oxidative stress-induced neuronal apoptosis in astrocytes, 6-shogaol reduces apoptosis by downregulating ROS, Bax and caspase 3 and by upregulating BDNF, GDNF, NGF, Bcl-2 and Bcl-xL via ERK1/2-mediated signaling pathway [343]. In another study with H_2O_2-treated HT22 hippocampal neuronal cells, 6-shogaol significantly increased choline acetyltransferase,the choline transporter and BDNF expression and reduced ROS production via the BDNF/TrkB-mediated signaling pathway [344]. Additionally, 6-shogaol has shown beneficial effects in LPS-treated BV2 and primary microglial cells by blocking NO, iNOS, PGE2, IL-1",TNF-α, Cox-2, P38 MAPK and NF-κB [345]. Furthermore, 6-shogaol provides a neuroprotective effect by attenuating Bax and promoting Bcl-2, Bcl-xL and BDNF in LPS- treated astrocytes [346]. In an *in vitro* model investigating the potential of 6-shogaol to mimic the neuritogenic activity of NGF in rat pheochromocytoma (PC-12) cells, the results demonstrated that treatment with 6-shogaol (500 ng/ml) exhibited neuritogenesis that was comparable to NGF (50 ng/ml) and was not cytotoxic to PC-12 cells, 6-shogaol induced low levels of NGF biosynthesis to induce neuritogenesis and also acted as a replacement for NGF over the activation of MEK/ERK1/2 and PI3K/AKT signaling pathways [347].

As there are no conclusive therapies for neuroinflammation and neuronal loss related to neurodegeneration. Therefore, the introduction of either the neurotrophic factor BDNF, or selected nutraceuticals for oxidative stress relief and neuroprotection could be a possible approach in this topic. In this topic, we have 2 research points: the first concerns the use of a new BDNF formula that can cross the blood-brain barrier and counteracts ROS-induced oxidative damage, the second concerns the study of the synergistic effects of curcumin, vitaminD3, and 6-shogaol combination as a potential dietary antioxidant supplement and evaluating the ability to increase BDNF expression as a means of neuroprotection.

2. Aims of the thesis

Since oxidative stress is considered the main contributor to all aging process including several related degenerative diseases, different combinations of essential bioactive molecules and natural products which could provide protection from unhealthy aging and could delay the onset and progression of degenerative diseases is investigated to propose them as dietary supplements These effects are analyzed on three different human tissues which are divided into three projects.

Project 1: Muscle aging, to test the effects of a combination of vitamin D3, curcumin and mineral in an *in vitro* model to support muscle activity and to provide protection against muscle loss during intensive hypercontraction.

Project 2: Retinae aging, to develop a new oral formulation able to counteract the early changes connected to glaucoma-related retinal degeneration using a combination of Gastrodin, vitamin D3, vitamin C, blackcurrant, and lycopene on *in vitro* and *ex-vivo* models.

Project 3: Brain aging, a combination of curcumin, vitamin D3 and 6-shogaol to provide neuroprotection inducing the production of brain derived neurotrophic factor (BDNF) after studying its important role on brain aging in an *in vitro* model.

3. Materials and Methods

3.1 Cell culture

In the experiments described in this thesis, different cell types were used, some primary cultures and other cell lines purchased from the American Type Culture Collection (ATCC), to study the effects of several compounds including bioactive molecules and natural extracts in the regulation of related processes. to aging in different tissues.

- **Project 1: Muscle aging**

C2C12 murine myoblasts, a subclone of the mouse myoblast cell line, purchased from the ATCC (Manassas, VA, USA) were chosen for their ability to differentiate rapidly and to form contractile myotubes producing characteristic muscle proteins [348]. This cell line was grown at 40-70% growth density without cell proliferation in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Milan, Italy), 100U/ml penicillin/streptomycin and maintained in an incubator at 37°C, 95% humidity and 5% CO_2. Depending on the various experimental protocols used, 1×10^4 cells were plated on 96-well plates to study the mitochondrial activity through MTT test and determining ATP level; 4×10^4 cells were plated in black 96-well plates to study oxygen consumption and mitochondrial membrane potential; 10×10^4cells/well were seeded in 6-well plates to investigate the activated intracellular pathways through western blot, to analyze the activation of TNFα by ELISA, and to determine the Glucose/Glycogen and lactate production.

- **Project 2: Retinal aging**

Retinal Ganglion Cells (RGCs) were extracted from 8-10-week-old mice C57BL/6JOlaHsd (n=108) based on a literature reported method [349; 350]. The animals were obtained directly from the enclosure of the University of Eastern Piedmont, selecting them from those who were not included in other experiments and not subjected to any treatment. This project received regular authorization with n. 04/2020-UT of 04/03/2020, pursuant to Legislative Decree 26/2014; issued by the General Directorate of Veterinary Health and Food-Ministry of Health (breeding plant: Department of Health Sciences located in via Solaroli, 17 Novara, obtained from the veterinary and animal welfare service of the Municipality of Novara, prot. num.

0087803/2017 of 13/12/2017). Briefly, eyeballs were extracted from the mouse's orbit along with a part of the optic nerve [351; 352]. The retina tissue deprived of the sclera was put into DMEM (Sigma-Aldrich, Milan, Italy) and was gently mechanically dissolved and centrifuged at 900 rpm for 10 min at room temperature. The pellet was resuspended in DMEM (Sigma-Aldrich, Milan, Italy) supplemented with 5% fetal calf serum (FCS, Sigma-Aldrich, Milan, Italy), 1% penicillin/streptomycin, and 2 mM L-glutamine (Sigma-Aldrich, Milan, Italy), and incubated at 37°C at 5% CO_2 and 95% humidity in incubator. After 72h the non-adherent cells were removed, and the culture medium was changed into a fresh one. Cells were expanded in the following days while maintaining their pigmentation for two weeks before starting the experiments till reaching 80% growth confluency [353]. Cells were plated in different manner based on the protocol experiments: 1×10^5 cells/well were plated in 96-well plate to study both cell viability by MTT test and ROS production; cells were plated in petri dishes till reaching 80% growth confluency to analyze the intracellular pathways by Western blot analysis. Before stimulation, the cells were maintained overnight in DMEM without red phenol (Sigma-Aldrich, Milan, Italy), supplemented with 2% FCS, 1% penicillin/streptomycin, and 2 mM L-glutamine in the incubator at 37 °C with 5% CO_2 and 95% humidity.

- **Project 3: Brain aging**

This project includes several different culture models, two primary cultures and three cell lines in order to explore many functions of molecules and natural extract to counteract the mechanisms that lead to brain aging and neurodegenerative diseases.

The Caco-2 cell line, an immortalized cell line of human colorectal adenocarcinoma, supplied by the American Type Culture Collection (ATCC), was cultured in Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM-F12, Merck Life Science, Rome, Italy) containing 10% FBS, 2 mM L-glutamine and 1% penicillin–streptomycin at 37 °C in an incubator at 5% CO_2. This cell line is used to carry out a widely accepted (by EMA and FDA) experimental model to predict absorption, metabolism and bioavailability of drugs and xenobiotics following oral intake. The cells were used at passage numbers between 26 to 32 to preserve the physiological balance between paracellular permeability and transport properties [354].

Human umbilical vein endothelial cells (HUVEC) were purchased from ATCC®. Cells were cultured in EGM Media (Lonza, Basel, Switzerland) supplemented with 10% FBS (Sigma-Aldrich, Milan, Italy), 1% penicillin/streptomycin (Sigma-Aldrich, Milan, Italy) and 2 mM Glutamine (Sigma-Aldrich, Milan, Italy) at 37 °C in a humidified atmosphere of 95% air, 5%

CO_2 [355]

Primary mouse astrocyte cultures were extracted from both male and female C57BL/6 mouse pups, following a classical technique as reported in literature [356] according to the National Guidelines for the Use and Care of Laboratory Animals. Briefly, within 24 h of birth, pups wereeuthanized, and cortices were dissected, minced, mechanically digested, and left to settle for 30 min at room temperature. Then, the cell suspension was centrifuged at 800 rpm for 5 min, pellets were resuspended in Neuronal Basal Medium (Sigma-Aldrich, Milan, Italy), supplemented with 5% FBS (Sigma-Aldrich, Milan, Italy), 1%penicillin/streptomycin (Sigma-Aldrich, Milan, Italy), and 2mM L-glutamine (Sigma-Aldrich, Milan, Italy), plated in multi-wells plates and maintained in culture for 6 days before treatment. Astrocytes should be separated from microglia and oligodendrocyte precursor cells by shaking, as reported in literature [357]. Cells were plated in different manner: 4×10^4 astrocytes/cm^2 were plated on a 24-well Transwell support with a polyester membrane with 0.4 μm pore size(Corning Costar, Sigma-Aldrich) to construct the blood brain barrier (BBB) model; 1×10^4 on 96-well plates for MTT assay and crystal violet staining; 4×10^4 cells were plated in black 96-well plates for oxygen consumption and mitochondrial membrane potential determination; 5×10^4 cells were plated on 24-well plates to analyze reactive oxygen species (ROS) and nitric oxide (NO) production; 2×10^5 cells were plated on 24-well plates to quantify BDNF by ELISA kit and determine BDNF extracellular/intracellular distribution; and 1×10^6 in 6-well plates to analyze both intracellular pathways by western blot and ERK activity by ELISA test. For the third part: 2×10^5 cells were plated on 24-well plates to quantify BDNF by ELISA kit and determine BDNF extracellular/intracellular distribution; and 1×10^6 in 6-well plates to analyze intracellular pathways by western blot.

Primary mouse cortical neuronal cells were obtained from the brains of C547BL/6 mouse pups (P0) as reported in literature [358]. All procedures used follow the guidelines in accordance with the National Institutes of Health Guidelines. Cortices were dissected from embryonic brains and the tissue was mechanically dissociated and left to settle for 30 min at room temperature then centrifuged. The supernatant was resuspended in Neuronal Basal medium (Sigma-Aldrich, Milan, Italy) supplemented with 2% B27 (Sigma-Aldrich, Milan, Italy), 1% penicillin/streptomycin (Sigma-Aldrich, Milan, Italy) and 2mM L-glutamine (Sigma-Aldrich, Milan, Italy). Cells were plated on pre-coated plates with 10µg/ml poly-L-lysine at a density of 1×10^6 cells/ml, maintained in an incubator at 37°C with 5% CO_2 and 95% humidity. Three days post plating, the medium was replaced, and the cells were maintained for 9-10 day in complete medium before the experiments. 1×10^4 cells were plated on 96-well plates to verify

cell viability by MTT test; 5×10^4 cells were plated on 24-well plates to analyze ROS production; 2×10^5 cells were plated on 24-well plates to quantify BDNF; 4×10^4 cells were plated in black 96-well plates to study oxygen consumption and mitochondrial membrane potential.

Human neuroblastoma SH-SY5Y cell line purchased from ATCC, is a well-known *in vitro* model often used to study neuronal function for its ability to differentiate into mature neurons [359]. These cells were cultured as previously described [360] in a DMEM/F-12 (Sigma-Aldrich, Milan) supplemented with 10% FBS (Sigma-Aldrich, Milan), 1% penicillin/streptomycin (Sigma-Aldrich, Milan), and grown in an incubator at 37°C at 95% humidity and 5% CO_2. The cells were plated for different experiments: 1×10^4 cells were plated on 96 well-plate to evaluate cell viability by MTT test, ROS and NO production, SOD3 activity, and amyloid precursor protein (APP) by ELISA test; 1×10^6 cells were plated on 6-well plates to analyze SIRT1, pTau, and β-amyloid by western blot.

3.2 Animal Experimental Model

To verify the obtained *in vitro* results, further experiments were carried out in *ex vivo* or *in vivo* specific models.

- **Project 2: Retinal aging**

Bovine eyecup preparation is a well-known ex vivo eye tissue model replicating the anterior chamber of the eye [361; 362] due to the high similarities between bovine and human visual systems as well as their retinal structure [363]. Retinae were isolated from bovine eyes [364; 365] obtained from the food industry and placed in physiological ice-cold saline solution supplemented with 1% penicillin/streptomycin for 2 h at 4°C. The retinal explants were maintained in red phenol free DMEM (Sigma-Aldrich, Milan, Italy) medium supplemented with 2% FCS, 3% penicillin/ streptomycin, and 2 mM L-glutamine in 6-well plates prepared with sterile foil to prevent retinal adhesion inside the incubator. The next day, the retinal explants were used for treatment with natural compounds.

- **Project 3: Brain aging**

12-month-old wild type C57BL/6jOlaHsd mice of comparable age to an elderly human (approximately 80 years old) [366] purchased from Envigo++++ (Bresso, Italy), were used to confirm the effects of BDNF solutions in a complex model (n=52). Using a new protocol of spontaneous intake [367], a new rissole prepared free of bromophenol blue containing 1.2 pg/ml BDNF SKA or 25 ng/ml BDNF was voluntarily eaten by old mice. The quantity of rissoles was calculated based on the quantity of daily food and water intake normally taken by the animals [368]. Rissoles were prepared in a sterile environment from pulverizing kibble food (Global Diet Envigo++++) mixed with 2.5 ml sterile water and 450 µl of BDNF solutions. The prepared food was wrapped in a heat sterilized foil to form a rissole and left to dry under the hood to maintain sterility for 18-24 h. Animals had full access to food and water, all experimental subjects were kept into a single cage housed in a constant temperature of 21-22°C, 5-55% humidity, for 3h [367, 369]. Later, the rissole was administered into the lower part of the cage, yet the mice had access to water and food in the upper part. The treatment period has started since administering the rissole during which the mice' health was monitored.

3.3 Experimental protocol

Since the aging project research includes several fields, the experimental protocol was subdivided following the specific area: muscles aging, retinae aging, and brain aging.

- **Project 1: Muscle aging**

C2C12 murine myoblasts were used to evaluate the effects of vitamin D3, magnesium bisglycinate, potassium citrate, and curcumin either alone or in combination to support the cellular mechanisms underlying exercise. In addition, the side effect of severe contraction caused by caffeine was investigated in presence of the most effective combination of the substances tested alone to prevent or restore the muscle damage. This study was divided into three phases. In the first phase, a dose-dependent study of single agents (24-72h) on cell viability was investigated to exclude negative effects. In particular, the concentrations of the substances were obtained from the literature: vitamin D3 (V) 0.001µM-1µM [370]; 15% magnesium bisglycinate chelate buffered (M) 0.1µM-5mM [371]; potassium citrate (K) 0.1mM-5mM [372]; curcumin (C) 1µM-100µM [373] were tested. The main effects observed were obtained by 1mM M and K, V100nM and C100µM, and these concentrations were used

together to verify the properties of a new combination named MKVC in successive phases. In the second phase, the main intracellular mechanisms underlying exercise were investigated in the presence of MKVC during physiological conditions. While in the third phase, the same analyses were carried out in the presence of caffeine 2.5mM, an agent that causes calcium release from the sarcoplasmic reticulum (SR) to mimic the effects of muscle contraction seen during an in vitro model of hypercontractility condition [374].

- **Project 2: Retinal aging**

RGC cells and retinal explants were used to study different biological effects involved in glaucoma in three phases. In phase one, cells and tissues were treated with agents (Vitamin D3, gastrodin, lycopene, vitamin C, and blackcurrant) alone and in combination (VGLCR) for 8 days to evaluate the effectiveness of the agents under physiological conditions [375] analyzing cell viability by MTT test. In addition, viability of RGC cells was analyzed periodically every 2 days for 10 days, to verify the correct timing of stimulation. In the second phase, RGC cells and retinal explants were pretreated with NMDA (300 µM) and H_2O_2 (200 µM) either separately or together for 4 days [376; 377] to mimic high pressure and oxidative damage, as occurs in human glaucoma disease, followed by the stimulation with VGLCR for 8 days [378] to verify the use of this combination under the pathological condition. In addition, the glaucoma inductors were also for 8 days after the presence of VGLCR for further 4 days, to study a possible protective mechanism exerted by the combination analyzing cell viability and ROS production. In addition, the main intracellular pathways involved in neuroinflammation, and oxidative stress were also investigated. In this model, exposure to inductors led to negative effects on retinal cells that were capable of simulating oxidative stress-related cellular degeneration, similar to what occurs in glaucoma, resulting in a realistic view of degenerative processes for 8 days [378]. Finally, in the third phase, the effectiveness of the combination (VGLCR) before and after the *in vitro* induction of glaucoma was verified on retinal explants analyzing the effects at the microscopic level and the main intracellular pathways involved in the early stages of the disease.

- **Project 3: Brain aging**

This research area includes different fields, which are described separately.

In the first one, the role of BDNF SKA was evaluated, both under physiological and oxidative stress conditions, using *in vitro* and *in vivo* models. Before treatment, both primary cortical neuronal cells and astrocytes were maintained in 0% FBS- red phenol free DMEM (Sigma-Aldrich, Milan, Italy), supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, Milan, Italy) 2mM L-glutamine (Sigma-Aldrich, Milan, Italy) and 1mM sodium pyruvate (Sigma-Aldrich, Milan, Italy) at 37°C, 5% CO_2 and 95% humidity for 1h. Cells were treated with 1pg/mlBDNF SKA and 50ng/ml BDNF at T0, checked every 24 h, and maintained for 6 days (namely 6 days treatment protocol); along with the vehicle, a saline solution was also used in treatment. Moreover, the involvement of TrkB was investigated using a specific antagonist ANA-12 (Sigma-Aldrich, Milan, Italy) which was added to cells at a concentration of 1µg/ml for 30 min before BDNF stimulation [379]. Additional experiments were also carried out to analyze the ability of BDNF solutions to restore the damage caused by oxidative stress as one of the major causes of aging and neurodegeneration. Both cortical neuronal cells and astrocytes were pre-treated with 200µM H_2O_2 (Sigma-Aldrich, Milan, Italy) for 30 min [380] followed with 1pg/ml BDNFSKA and 50ng/ml BDNF stimulations. Finally, astrocytes were used in a 24 h to mimic human posology. In *in vivo* experiments, animals were divided into four different treatment period groups: Untreated (12 animals, 4 animals for each treatment period), 24 h group (20 animals), 24 h plus 24 h group (10 animals), and 6-day protocol group (10 animals, only one administration left for 6 days). At the end of each time point the animals were sacrificed by CO_2 asphyxiation according to treatment groups: 24 animals for 24 h treatment group, 14 animals of 48h treatment group (24 h with rissole plus 24 h without rissole), 14 mice of the 6 days protocol group were sacrificed at the end of the 6 days. In addition, blood was collected by intracardiac withdrawal, and brain tissues were collected after the animal's sacrifice. Blood was centrifuged at 3500 rpm for 15 min at room temperature and the serum was conserved at -80 °C for subsequent experiments. Brain was removed, frozen and conserved at -80 °C for following analyses by ELISA and Western blot.

In the other one, SH-SY5Y cell line was used to study the effects of Curcumin, Vitamin D3 and 6-shogaol, alone and combined, to study two different biological aspects involved in brain aging and neurodegeneration: oxidative stress and iron-dependent damage. In the first set of experiments, preliminary screening of curcumin (5µM-20µM) [381], Vitamin D3 (0.1 nM-10 nM) [382], and 6-shogaol (5µM-20µM) [3832] was performed in a dose/response in time-

course study, analyzing cell viability, to determine the optimal concentration, and then the concentrations (Cur 20µM, VitD3 1nM and 6-shogaol 5µM) were maintained in all successive experiments. The combination between Cur 20µM, VitD3 1nM and 6-shogaol 5µM (named CVS) was investigated for 24 h to determine the efficiency of the new formula compared to single agents. In the second set of experiments, the effect of curcumin, vitamin D3 and 6-shogaol, alone and combined, were tested to counteract oxidative stress; in this context, cells were pretreated for 30 min with 100µM H_2O_2 [384]. Particularly, the ability of Cur 20µM, VitD3 1nM and 6-shogaol 5µM, alone and combined to prevent or restore the damage caused by oxidative stress, was analyzed by the MTT test. Moreover, ROS and NO production, amyloid precursor protein (APP) quantification, SOD3 activity, and Western blot analysis were also performed at 24 h. In a third set of experiments, to induce neurodegeneration, cells were pretreated with catalytic iron (Fe^{3+}) 75µM [382] for 24 h and then treated with Cur 20µM, VitD3 1nM and 6-shogaol 5µM, alone and combined, for additional 24 h to investigate the ability of compound to restore the damage, analyzing cell viability, ROS and NO production, SOD3 activity, APP quantification, and the main intracellular pathways involved.

Based on the results obtained from SH-SY5Y, additional experiments were performed in order to verify if CVS was able to induce BDNF. Since neurons need the presence of astrocytes to make more physiological functions, the astrocytes were treated with Cur 20µM, VitD3 1nM and 6-shogaol 5µM, alone and combined, and the conditioned medium was used to treat SH-SY5Y cells for 24 h [385] which were pretreated for 30 min with 100µM H_2O_2 or for 24 h with 75µM Fe^{3+} in order to mimic the neurodegenerative process [386]. BDNF production was quantified using ELISA kit, analyzing the concentration of BDNF on extracellular and intracellular environments.

3.4 Agents' preparations

- **Project 1: Muscle aging**

C2C12 cells were treated with different concentrations of vitamin D3 (V) 0.001µM-1µM [370]; 15% magnesium bisglycinate chelate buffered (M) 0.1µM-5mM [387]; potassium citrate (K) 0.1mM-5mM [372]; curcumin (C) 1µM-100µM [373]. The substances were dissolved directly in 0% FBS DMEM without red phenol supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate. The effects of vitamin D3, magnesium bisglycinate, potassium citrate, and curcumin were tested also in the presence high concentration of caffeine

(2.5 mM) prepared in 0% FBS DMEM without red phenol and supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate.

- **Project 2: Retinal aging**

Both cells and tissues were treated with vitamin D3 (V, 100 nM) [388], lycopene (L, 20 µM) [389], vitamin C (C, 200 µM) [390], and blackcurrant extract (R, 200 mM) [391] following the methods reported in the literature. Gastrodin (G), on the other hand, was previously tested in a dose-response study ranging from 0.1 µM to 100 µM to verify the most effective concentration on RGC, and then the 50 µM dose was used in retinal explants [392]. All of these agents were dissolved in FM-LipoMatrix®, a new technology based on a patented solvent (patent N°102017000036744 by noiVita srls, produced by Pro-Bio INTEGRA srl, Rovigo, Italy), and were tested alone and in combination (in this work the compound was referred to as VGLCR) following the hypothesis of a new potential formula to be used in humans. The effects of VGLCR were studied before or after experimental induction of glaucoma by means of N-methyl-D-aspartate (NMDA, 300 µM; Sigma-Aldrich, Milan, Italy) and H_2O_2 (2 mM; Sigma-Aldrich, Milan, Italy) on both cells and tissues, since NMDA and H_2O_2 are important well known glaucoma inductors [393] capable of mimicking high pressure and oxidative damage, as occurs in humans. Glaucoma inductors were dissolved in DMEM without red phenol and FBS, supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate.

- **Project 3: Brain Aging**

All dilutions were prepared from a BDNF 4CH stock solution (0.001ng/ml) in 0.9% NaCl. According to previous studies on activated blends [394; 395] BDNF solution was prepared at a concentration of 1pg/ml using the sequential kinetic activation (SKA) method [394]. The solution was kinetically energized by a mechanically applied force via a standardized vertical shaking process corresponding to 100 oscillations in 10s (sequential kinetic activation, SKA), BDNF solution was prepared by GUNA Laboratories (GUNA S.p.a, Milan, Italy). The volume of BDNF solution was calculated by comparing the volume added to the sample treated with 50 ng/ml BDNF [396]. The BDNF used to compare the results obtained with BDNF 1pg/ml SKA was not subjected to SKA treatment to replicate the same experimental conditions as in other studies.

SH-SY5Y were treated with Curcumin, Vitamin D3 and 6-shogaol, alone and combined, dissolved in DMEM without red phenol with 0.5%FBS, supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate.

3.5 Cell viability

The MTT test, based on the reduction of water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into insoluble formazan crystals was used to exclude any cytotoxic effects of each selected compound. MTT-based In Vitro Toxicology Assay Kit (Sigma-Aldrich, Milan, Italy) was performed on a 96-well plate to determine cell viability after each stimulation as previously described [397]. At the end of stimulation, cells were incubated with 1% MTT dye for 2 h in an incubator at 37°C, 5% CO_2 and 95% humidity. Later, the purple formazan crystals were dissolved in equal volume of MTT Solubilization Solution. Cell viability was determined by measuring the absorbance at 570 nm with correction at 690 nm, through a spectrometer (VICTOR X4, multilabel plate reader), and calculated by comparing results to the control (0% value). This test was performed **in all projects (from 1 to 3)**.

3.6 *In vitro* intestinal barrier model

Since most of the substances tested includes molecule and natural extract use in humans like a nutraceutical source, additional tests were carried out to verify their intestinal absorption after oral intake using a Transwell model able to mimic the passage of substances to blood vessels (particularly in **Project 3**).

2×10^4 Caco-2 cells [397; 398] were seeded on the apical side of 24-well Transwells with polyester membrane of 0.4 µm pore size (Corning®, Sigma-Aldrich, Milan, Italy) in complete medium, maintained in culture for up to 21 days in a humidified incubator at 37°C with 5% CO_2 and 95% humidity, medium is changed every 3 days first basolateral and then apical during which monolayer integrity was checked [399]. 21 days later, 1pg/ml BDNF SKA and 50 ng/ml BDNF were added to culture medium under different pH conditions [399]; pH 6.5 in the apical side represents the average pH in the lumen of the small intestine, whereas pH 7.4 in the basolateral side represents blood pH. During the treatment course, cells were maintained in an incubator at 37°C under 95% humidity and 5% CO_2. To understand the ability of tested substances to cross the intestinal barrier, the medium in the basolateral part was collected (from 30 min to 6 hours) and BDNF was quantified at each time point using an ELISA kit.

3.7 *In vitro* Blood Brain Barrier (BBB) model

Astrocytes were co-cultured with HUVEC cells according to methods reported in literature [400]. HUVEC cells, purchased from ATCC®, were cultured in EGM Media (Lonza, Basel, Switzerland) supplemented with 10% FBS (Sigma-Aldrich, Milan, Italy), 1% penicillin/streptomycin (Sigma-Aldrich, Milan, Italy) and 2mM L-glutamine (Sigma-Aldrich, Milan, Italy) in an incubator at 37°C under 95% humidity and 5% CO_2. Briefly, to create a blood brain barrier (BBB), 4×10^4 astrocytes/cm^2 were plated on the basolateral side of flipped 6.5 mm Transwells with polyester membrane of 0.4 µm pore size (Corning®, Sigma-Aldrich, Milan, Italy) and left to attach for 4h. Transwells were later placed into the normal orientation and cells were left to grow for 48h. Later, 1×10^5 HUVEC cells/cm^2 were plated in the apical compartment. The inserts were then placed in a 24-well plate. After 7 days of culture, transwells were treated and permeability studies were performed [401]. To understand the ability of tested substances to cross the blood-brain barrier, samples from the basolateral side of Transwells were collected over 1-6 hours and 30 min for BDNF quantification. This test was performed in **Project 3.**

3.8 Crystal violet staining

Astrocytes were fixed with 1% glutaraldehyde (Sigma-Aldrich, Milan, Italy) for 15 min at room temperature, then washed and stained with 100 µl 0.1% aqueous crystal violet (Sigma-Aldrich, Milan, Italy) for 20 min at room temperature, finally mixed using 100µl of 10% acetic acid before reading the absorbance at 595 nm using a spectrometer (VICTOR X4, multilabel plate reader). The estimated number of cells was calculated by comparing the results to the control cells counted at T0 [402]. This test was performed in **Project 3.**

3.9 ROS production

The rate of reactive oxygen species (ROS) production was measured using a standard protocol based on superoxide dismutase-inhibitable reduction of cytochrome C (Sigma-Aldrich, Milan, Italy) [382]. In brief, 100 µl cytochrome C (Sigma-Aldrich, Milan, Italy) was added to all samples (treated and untreated), along with another sample containing 100 µl superoxide dismutase only (Sigma-Aldrich, Milan, Italy), and were kept for 30 minutes in an incubator at 37°C. The absorbance was measured at 550 nm using a spectrometer (VICTORX4, Multilabel Plate Reader), the resulting O_2 was expressed as mean ± SD (%) nanomoles per reduced

cytochrome C per microgram protein compared to control. This test was performed in **projects 2 and 3.**

3.10 SOD activity

The Superoxide Dismutase Assay Kit assay kit was used to measure all three types of SOD enzymes (Cu/Zn, Mn, and FeSOD) (Cayman Chemical company, Ann Arbor, Michigan, United States) according to the manufacturer's instructions. In a 96-well plate, 200 µl of the diluted Radical Detector was added to 10 µl of each sample and at the same time a standard curve was generated (0.05-0.005 U/ml). Then, 20 µl of diluted Xanthine Oxidase was added and the plate was put on a microplate shaker to mix the contents for 30 minutes. Finally, the absorbance was measured at 480 nm using a spectrometer (VICTORX4, multilabel plate reader). Results were expressed as means (%) compared to control [403]. This test was performed in **projects 2 and 3.**

3.11 Griess Assay

Nitric Oxide (NO) production was measured by Griess Assay (Promega Corporation, Madison, Wisconsin, United States), the supernatant of each sample was mixed with equal volumes of Griess reagent and incubated in the dark at room temperature for 10 min. The absorbance was measured at 570 nm by a spectrometer (VICTORX4, multilabel plate reader) and the NO production corresponded to the NO (µmol) produced after each stimulation, each containing 1.5 µg of protein [404]. This test was performed in **project 3.**

3.12 Oxygen consumption and mitochondrial membrane potential

Both oxygen consumption and mitochondrial membrane potential were analyzed following manufacturer's instructions in oxygen consumption/Mito membrane potential Dual Assay Kit (Cayman Chemical company, Ann Arbor, Michigan, United States) [402]. In brief, 10 µl of the JC-1 staining solution was added to cells treated/non treated, followed by a 15–30-minute incubation at 37 °C, 5% CO_2 and 95% humidity. The samples were centrifuged for 5 minutes at 400 xg at room temperature and the supernatant was aspirated. Next, 200 µl of Assay Buffer was added to the samples, centrifuged for 5 minutes at 400 xg at room temperature and the supernatant was aspirated (this step was repeated at least 5 times). Finally, 100 µl of Assay buffer was added to each well, and the mitochondrial membrane potential was measured using JC-1 aggregates at an excitation/emission of 560/590 nm for healthy cells and monomers at an

excitation/emission of 485/535 nm for apoptotic cells using a fluorescence spectrometer (VICTOR X4, multilabel plate reader). Results are expressed as means ± SD (%) compared to control cells. This test was performed in both **projects 1 and 3**.

3.13 ATP assay

At the end of each stimulation, cells were treated with the components of the ATP assay kit (Calbiochem, San Diego, USA) following the manufacturer's instructions. Briefly, cells were lysed with ice-cold Ripa buffer and centrifuged at 14000 rpm at 4°C for 20 min, supernatants were collected. To each sample was added the components of the ATP assay kit (nucleotide releasing buffer, ATP, enzyme reconstitution buffer, ATP monitoring enzyme). Luminescence was measured 1 min after adding ATP monitoring enzyme using a spectrometer (VICTOR X4, multilabel plate reader). The total protein in each sample was measured by BCA assay (Thermo Fisher Scientific, Waltham, USA), and luminescence was calculated as µmol ATP/g protein and reported as means ± SD of nanomole/well [405]. This test was performed in **project 1**.

3.14 p53 activity

p53 activity was measured by a specific ELISA kit (Cayman Chemical company, Ann Arbor, Michigan, United States) directly on nuclear extracts obtained following the manufacturer's instructions, as also reported in the literature [406]. Briefly, cells were lysed with ice-cold 1X Complete Hypotonic Buffer, supplemented with NP-40 and centrifuged at 12,000 xg at 4 °C for 10 min. The pellet was solubilized with ice-cold Complete Nuclear Extraction Buffer 1X, supplemented with protease and phosphatase inhibitors followed with another centrifuge cycle at 12,000 xg for 15 min at 4 °C. The supernatant was examined to analyze the activity of p53 in accordance with the protein quantification through the BCA assay (Thermo Fisher Scientific, Waltham, USA). This test was performed in **projects 2 and 3**.

3.15 ERKs activity

ERK/MAPK activity was determined by the InstantOne™ ELISA (Thermo Fisher Scientific, Waltham, USA) on cell lysates following the manufacturer's protocol [403]. Briefly, cells at the end of treatments were lysed with 100 μl Cell Lysis Buffer Mix, 50 μl/well of each sample was loaded in InstantOne ELISA microplate strips, to which the Antibody Cocktail was added and incubated for 1 h at room temperature on a microplate shaker. The Detection Reagent was

added later and after 20 min, the reaction was stopped by the stop solution. The absorbance was measured at 450 nm by a spectrometer (VICTOR X4, multilabel plate reader). The results were expressed as mean absorbance (%) compared to the control (reported in **projects 2 and 3**).

3.16 $[Mg^{2+}]i$ movements

Intracellular Magnesium concentration ($[Mg^{2+}]i$) was measured using Mg^{2+}-sensitive fluorescent dye Mag-fura-2AM (Furaptra, Biotium). Briefly, cells were incubated at 37°C for 30 min in a Hanks salt solution free of Mg^{2+} (10 mM glucose, 20mM Hepes/Tris pH 7.4, 1.3 mM $CaCl_2$, 5µM Mag-fura-2AM) (Thermo Fisher Scientific, Waltham, USA). Mag-fura-2Am fluorescent-loaded cells were monitored at regular intervals 3 min-300 min at excitation/emission of 340 nm and 380/510 nm with an exposure of 100 ms using a Fluorescence spectrometer (VICTOR X4, multilabel plate reader). After background subtraction, fluorescence ratios (340/380 nm) were calculated and compared to control; Rmax and Rmin were analyzed after the addition of 50 mM $MgCl_2$ and 100 mM EDTA respectively [407]. This test was performed in **project 1.**

3.17 $[Ca^{2+}]$ movements

$[Ca^{2+}]$ movements during the contraction/relaxation cycle were determined using Fura-2AM (Sigma-Aldrich, Milan, Italy). After the end of stimulation, supernatant was removed, and cells were twice washed with sterile PBS 1X then incubated with 5µM Fura-2AM in PSS buffer free of Ca^{2+} (1.5 mM KCl, 10mM HEPES, 10mM D-Glucose, 2mM L-glutamine, pH 7.4) in dark for 30 min in agitation at 37°C. Fluorescence was measured at excitation/emission of 340 nm/510 nm using a fluorescence spectrometer (VICTOR X4, multilabel plate reader). Results were reported as means ± SD (%) compared to control cells [408]. This test was performed in **project 1.**

3.18 TNF-α assay

TNF-α concentration was determined according to the TNF-α ELISA kit (Sigma-Aldrich, Milan, Italy), analyzing 100µl of each sample on a 96-well ELISA plate. The plate was incubated for 2h at room temperature followed by an overnight incubation at 4°C. Next, the wells were washed and incubated with 100 µl of biotinylated anti-TNF-α for 2 hours at room temperature, then followed with another wash and 1 hour incubation with 100 µl Streptavidin-HRP. Later, 100 µl of chromogen solution was added and incubated for 30 minutes incubation

at room temperature. Finally, the absorbance was measured at 450 nm after adding the stop solution using a spectrometer (VICTOR X4, multilabel plate reader) [409]. This test was performed in **project 1.**

3.19 Glucose uptake

Glucose uptake was determined following the manufacturer's instructions by Glucose Uptake Colorimetric Assay Kit (Sigma-Aldrich, Milan, Italy). Cells were lysed using the extraction buffer under several cycles of freezing/thawing and then heated at 85°C for 40 min. The resulting cooling cell lysates were placed in ice for 5 minutes, neutralized by 10 µl Neutralization Buffer, centrifuged at 13000 xg to remove any insoluble material. The amount of glucose in cell lysate was measured after reacting with a mixture of Reagents A & B found in the kit. The absorbance was measured at 412 nm by a spectrometer (VICTOR X4, multilabel plate reader), data was reported as means ± SD of pmol/µl derived from standard curve [410]. This test was performed in **project 1**.

3.20 Glycogen measurement

The Glycogen synthesis was determined following the manufacturer's instructions (Bio Vision, Life Research, Scoresby Victoria, Australia) directly on cells lysed, which were boiled for 5 minutes for enzyme inactivation. Samples were centrifuged at 13000 rpm for 5 min, supernatants were collected to be used to induce glycogen hydrolysis into glucose in the presence of OxiRed probe to generate color, the absorbance was measured at 570 nm using a spectrometer (VICTOR X4, multilabel plate reader), the glycogen concentration in each sample was calculated according to the following equation C=Ay/Sv, where Ay is the amount of glycogen (mg) in the sample according to the standard curve and Sv is the sample volume (ml) [411]. Data was reported as means ± SD (pg/µl) vs control. This test was performed in **project 1.**

3.21 Lactate measurement

The lactate level was quantified according to the manufacturer's protocol by Lactate assay Kit (Bio Vision, Life Research, Scoresby Victoria, Australia) [412] on cells which were lysed in Lactate assay Buffer and centrifuged at 13000 xg for 10 minutes to remove insoluble components. Lactate concentration was determined after adding a master reaction mix (lactate assay buffer, lactate enzyme mix and lactate probe), incubated for 30 minutes in the dark at room temperature. Absorbance was measured at 570 nm using a spectrometer (VICTOR X4,

multilabel plate reader). The concentration of lactate in each sample was determined according to the following equation C=Sa/Sv, in which Sa is the amount of lactate acid in an unknown sample from the standard curve, Sv is the sample volume. Followed by multiplying with the lactate molecular weight= 89.07 g/mole. Data was reported as means ± SD (mmol/dl). This test was performed in **project 1.**

3.22 Akt activation assay

PI3K/Akt activities were determined using The InstantOne TM ELISA kit (Thermo Fisher Scientific, Waltham, USA) [403]. According to the manufacturer's protocol, 50 µl/well of cell lysed sample was added in InstantOne ELISA microplate strips containing antibody cocktail for 1 hour at room temperature on a microplate shaker. Next, the detection reagent was added for 20 minutes and finally the reaction was stopped after the addition of a stop solution. The absorbance is measured at 450 nm using a spectrometer (VICTOR X4, multilabel plate reader), results were presented as mean absorbance (%) ± SD compared to control. This test was performed in **project 1**.

3.23 Phospho-p38/MAPK ELISA test

Phosphorylation levels of p38/MAPK were analyzed following the manufacturer's protocol of ELISA kit (p38 MAPK alpha (pT180/pY182) + total p38 MAPK alpha ELISA Kit, Abcam). As follows, cells were lysed using the kit cold Lysis buffer supplemented with protease and phosphatase inhibitors. Supernatants were added to corresponding plate strips to which an antibody cocktail was added to each sample, plate was incubated for 1 hour at room temperature on a plate shaker. Next, a washing step was performed followed by adding TMB substrate for 15 min in the dark on a microplate shaker. Finally, the reaction was stopped, and the absorbance was read at 450 nm using a spectrometer (VICTOR X4, multilabel plate reader). Results were presented as means absorbance (%) ± SD vs control [413]. This test was performed in both **projects 1 and 2**

3.24 JNK activity

JNK activity was measured on cells and tissue homogenates using the Abcam JNK1/2 (pT183/Y185) +Total JNK1/2 ELISA kit (abcam CN 176662) following the manufacturer's instructions [414]. Briefly, both 50 µL samples and 50 µL of Antibody Cocktail were incubated at room temperature for 1 hour. After 3 times washing with wash buffer PT 1X, 100 µL of TMB substrate was added and incubated for 15 min; at the end, 100 µL of stop solution was

used to stop the reaction. Absorbance was measured at 450 nm using a spectrometer (VICTOR X4, Multilabel Plate Reader). Data were expressed as mean (%) ± SD compared to control (**project 2**).

3.25 MMP-9 quantification assay

MMP-9 ELISA Kit (DBA Italia, Milan, Italy) was used to quantify MMP-9 protein in samples supernatant as follows in the manufacturer's instructions [394]. Briefly, samples' supernatants added into a 96-well plate were incubated at 37 °C for 90 min, later both biotinylated antibody and ABC working solution were added and the plate was incubated at 37 °C for 60 and 30 min, respectively. TMB substrate was added and incubated for 15 min at 37 °C, finally TMB stop solution was added. The MMP-9 concentration was determined after measuring the absorbance at 450 nm using a spectrometer (VICTOR X4, Multilabel Plate Reader) and calculated by comparing results to the MMP-9 standard curve. This test was performed in **Project 2**.

3.26 Quantification of retinal layer

The pictures of the retinal explants at 0 and 8 days either treated or non-treated were taken using Nikon D70 camera, and the areas were quantified using ImageJ program [415], as reported in **project 2**. The retinal areas after each stimulation were calculated and compared to both pictures taken at day 0 (T0, area 100%) and the untreated sample (control). The results were expressed as a percentage of the relative reduction in the under-study area compared to the original size, like that used for the wound healing area measurement using the following formula [394;416]:

% area = [(WA0 - WA)/WA0] × 100

WA0 = original size of retinal explanted

WA = area after each stimulation

3.27 Brain-derived neurotrophic factor (BDNF) quantification

Brain-derived neurotrophic factor (BDNF) was quantified by Rat BDNF Elisa Kit (Thermo Scientific ™, Waltham, MA, United States) in cellular supernatants obtained from the basolateral part of BBB *in vitro* model, primary cortical neuronal cells, and astrocytes; in addition, extracellular and intracellular BDNF concentration of both astrocytes and human neuroblastoma-astrocytes was carried out. Briefly, cells were washed and lysed in saline solution and all samples were incubated into the ELISA microplate strips overnight at 4°C with

gentle shaking, followed by a series of washing with 1X Wash Buffer repeated before each step. Next, 100 µl of biotinylated conjugate was added and incubated at room temperature for 1 hour with gentle shaking. 100 µl of prepared Streptavidin-HRP solution was added and incubated at room temperature for 45 minutes with gentle shaking. Later, 100 µl of TMB solution was added and incubated for 30 minutes in the dark with gentle shaking. Finally, the reaction was stopped with 50 µl of Stop Solution and the absorbance was read at 450 nm within 30 minutes after adding the Stop Solution using a spectrometer (VICTOR X4, multilabel plate reader). BDNF concentration was obtained using a generated standard curve and expressed as mean absorbance (%) compared to the control [417]. In addition, brain tissue samples along with serum and brain tissue lysates were collected from animal models to quantify BDNF. Briefly, 100 mg tissue of brain tissues was homogenized with 300 µl cold lysis buffer (0.1 M Tris, 0.01 M NaCl, 0.025 M EDTA, 1% NP-40, 1% Triton X-100) supplemented with 2 mM sodium orthovanadate, 0.1 M sodium fluoride, 1:100 protease inhibitors mix (all purchased from Sigma-Aldrich, Milan, Italy) using an electric potter at 1600 rpm for 2 minutes, and the tissue extracts were centrifuged at 13000 rpm for 20 min at 4℃. Supernatants were collected for BDNF quantification (**project 3**).

3.28 Amyloid Precursor Protein (APP) Quantification

Amyloid precursor protein (APP) quantification was measured by the amyloid Beta A4 protein ELISA Kit (Sigma-Aldrich, Milan, Italy) on cellular supernatants following the manufacturer's instructions [386]. Briefly, after the end of treatment period, cellular supernatants were collected and loaded into a 96-well plate that the biotinylated detection antibody specific for the target protein was added to each well and incubated for 1 hour at room temperature, followed with a 45-minute incubation with HRP-conjugated streptavidin. Next, a TMB substrate solution was incubated for 30 minutes, and finally the reaction was stopped by a stop solution. The absorbance was measured at 450 nm by a spectrometer (VICTOR X4, multilabel plate reader), APP concentration was determined according to the plotted APP standard curve **(Project 3**).

3.29 Western blot

In order to analyze the intracellular pathways activated by the selected antioxidants during aging, Western blot analysis was performed followed by densitometric analyses in all 3 projects.

- **Project 1**

At the end of treatments, C2C12 cells were twice washed with cold-PBS 1X and lysed in ice using a lysis buffer consisting of RIPA buffer (50 mM Hepes, 150 mM NaCl, 0.1% SDS, 1% Triton 100X, 1% deoxycholate acid, 10% glycerol, 1.5mM $MgCl_2$, 1 mM EGTA, 1mM NaF) supplemented with 2mM sodium orthovanadate and 1mM phenylmethanesulfonyl fluoride (PMSF) and 1:100 mix Protease Inhibitor Cocktail (all purchased from Sigma-Aldrich, Milan, Italy). 40 µg protein from each sample was resolved into 8% and 15% SDS-PAGE gels and transferred onto Polyvinylidene difluoride (PVDF) membranes (GE Healthcare Europe GmbH, Milan, Italy) which were incubated overnight at 4°C with specific primary antibodies as follows: anti-rabbit Cyclin D1 (1:1000, cell signaling), anti-mouse Desmin (1:1000, Santa-Cruz), anti-rabbit Phospho-AMPK (1:1000, Millipore), anti-mouse AMPK (1:500, Santa-Cruz), anti-mouse Phospho-JNK (1:500, Santa-Cruz), anti-mouse JNK (1:500, Santa-Cruz) and anti-mouse SMA (1:1000, Santa-Cruz). Protein expression was normalized and verified by anti-mouse β-actin (1:5000, Sigma-Aldrich) and reported as mean ± SD (%) vs control.

- **Project 2**

After stimulation, RGC cells were lysed on ice with Complete tablet buffer (Roche, CN 11836145001), supplemented with 2 mM sodium orthovanadate, 0.1 M sodium fluoride, 1:1000 phenylmethanesulfonyl fluoride and 1:100 mix protease inhibitor cocktail (All purchased from Sigma-Aldrich, Milan, Italy). In total, 35 µg protein from each lysate was resolved on 10% SDS-PAGE gels and transferred onto PVDF membranes which were incubated overnight at 4°C with a specific primary antibody: anti-Annexin V (1:1000, Santa Cruz, sc-74438), anti-iNOS (1:1000, Santa Cruz, sc-7271), and anti-Bax (1:250, Santa Cruz, sc-7480).

In addition, retinal explants were plated in a 60 mm dish, and after stimulation were lysed in ice that 100 mg tissue was homogenized with 150 µL of lysis buffer (0.1 M Tris, 0.01 M NaCl, 0.025 M EDTA, 1% NP-40, 1% Triton X100, Sigma-Aldrich, Milan), supplemented with 2 mM sodium orthovanadate, 0.1 M sodium fluoride, 1:100 mix of protease inhibitors, 1:1000 phenylmethylsulfonyl fluoride, using an electric potter at 1600 rpm for 2 min. Samples were mixed for 30 min at 4°C, centrifuged for 30 min at 13000 rpm at 4°C. Next, 40 µg proteins

were resolved on 10% SDS-PAGE gels and transferred onto PVDF membranes which were incubated overnight at 4°C with a specific primary antibody: anti-Bax (1:250, Santa Cruz, sc-7480), anti-iNOS (1:1000 Santa Cruz, sc-7271), anti-SIRT1 (1:1000, Sigma-Aldrich), and anti-OPA1 (1:250, Santa Cruz, sc-393296). Protein expressions of both RGC and retinal lysates were normalized and verified through anti-β-actin detection (1:5000, Sigma-Aldrich). Results were expressed as a mean ± SD (%) vs control.

- **Project 3**

After stimulation with BDNF, astrocytes were lysed in ice with Ripa Buffer (50 mM Hepes, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate acid, 10% glycerol, 1.5 mM MgCl2, 1 mM EGTA, 1 mM NaF) supplemented with 2 mM sodium orthovanadate and 1:100 mix Protease Inhibitor Cocktail. 30 µg proteins were resolved on 8% and 15% SDS-PAGE gels, transferred onto PVDF membranes which were incubated overnight at 4°C with specific primary antibodies: anti-phospho-tyrosine receptor kinase B (p-TrkB, Tyr515; 1:250, Santa Cruz, CA, United States), anti-tyrosine receptor kinase B (trkB; 1:250, Santa Cruz, CA, United States), anti-Apolipoprotein E (apoE, E4; 1:250, Santa Cruz, CA, United States), anti-phospho-Sirtuin1 (pSIRT1, Ser47; 1:1000, Sigma-Aldrich, Milan, Italy), anti-Phospho-p44/p42 Mitogen-activated protein kinase (pERK/MAPK, Thr202/Tyr204; 1:1000, Euroclone, Milan, Italy), anti-p44/p42 Mitogen-activated protein kinase (ERK/MAPK; 1:1000, Euroclone, Milan, Italy) and anti-Phospho-Tau (pTau, Ser262; 1:250, Thermo Fisher Scientific, Waltham, MA, United States). In addition, brain tissue was excised out, washed in ice saline solution, weighed, cut in small pieces, each 100 mg tissue was homogenized with 300 µl with cold lysis buffer (0.1 M Tris, 0.01 M NaCl, 0.025 M EDTA, 1% NP-40, 1% Triton X-100) supplemented with 2 mM sodium orthovanadate, 0.1 M sodium fluoride, 1:100 mix of protease inhibitor cocktail, and 1:1000 phenylmethylsulfonyl fluoride (PMSF) (all purchased from Sigma-Aldrich, Milan, Italy) using an electric potter at 1600 rpm for 2 min. the brain tissue lysates were centrifuged at 13000 rpm for 20 min at 4°C, and 40 µg proteins were resolved on 8% and 15% SDS-PAGE gels, then transferred onto PVDF membranes which were incubated overnight at 4°C with specific primary antibodies: anti-BDNF (1:500, Sigma-Aldrich, Milan, Italy) and anti-β-Amyloid (APP, B-4, 1:500, Santa Cruz, CA, United States) were also investigated.

SH-SY5Y and Astrocytes were lysed in ice with Ripa Buffer (50 mM Hepes, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate acid, 10% glycerol, 1.5 mM MgCl2, 1 mM EGTA, 1 mM NaF) supplemented with 2 mM sodium orthovanadate and 1:100 mix Protease Inhibitor Cocktail after stimulation time with Cur 20µM, VitD3 1nM and 6-shogaol 5µM**,** 30

µg proteins were resolved on 8% and 15% SDS-PAGE gels, then transferred onto PVDF membranes which were incubated overnight at 4°C with specific primary antibodies: anti-tyrosine receptor kinase B (trkB; 1:250, Santa Cruz), anti-Phospho-Tau (pTau, Ser262; 1:250, Thermo Fisher Scientific), anti-β-Amyloid (APP, B-4, 1:500, Santa Cruz), anti-pro-BDNF (pro-BDNF; 1:500, Santa Cruz), anti-SIRT1 (1:1000, Sigma-Aldrich). All protein expressions were normalized and verified through β-actin detection (1:5000, Sigma-Aldrich, Milan, Italy) and expressed as mean ± SD (%) vs control.

3.30 Statistical analysis

In vitro results from at least 4 independent experiments were processed using Prism GraphPad statistical software for normalization, analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test to compare percentages of responses. Multiple comparisons between groups were followed by a two-sided Dunnett post hoc testing. All results were expressed as mean ± SD, $p < 0.05$ was considered statistically significant **(Projects 1,2 and 3)**. Images produced during **project 2** were obtained by BFBZ and ImageJ. Data collected from the *in vivo* model from 4 independent experiments were analyzed by two-way ANOVA followed by Bonferroni post hoc test to compare percentages of responses. Comparisons between the two groups were performed using a two-tailed Student's t-test. Multiple comparisons between groups were followed by a two-sided Dunnett post hoc testing. All results were expressed as mean ± SD, $p < 0.05$ was considered statistically significant (**Project 3**).

4. Results

In order to explore the effect of biomolecules and natural extracts with antioxidant action in the modulation of aging processes, the results obtained were collected according to the order of execution of the projects' order (from 1 to 3). All the results described below have been published in a peer-reviewed international scientific journal.

4.1. Project 1: Muscle aging

Data obtained support the initial hypothesis of the project, underline the key role of Vitamin D3, and Curcumin associated with mineral salts in maintaining muscular health.

Dose-response and time-dependent study of cell viability on C2C12 cells

In order to verify the biological efficiency and exclude any cytotoxic effects, C2C12 cells were stimulated with magnesium (M), potassium citrate (K), vitamin D3 (V) and curcumin (C) alone in dose-response and time-dependent experiments (24-72 h) by analyzing cell viability using MTT assay. Moreover, these experiments were important to understand the best concentration to use to create a new formulation able to improve age-related muscle decay.

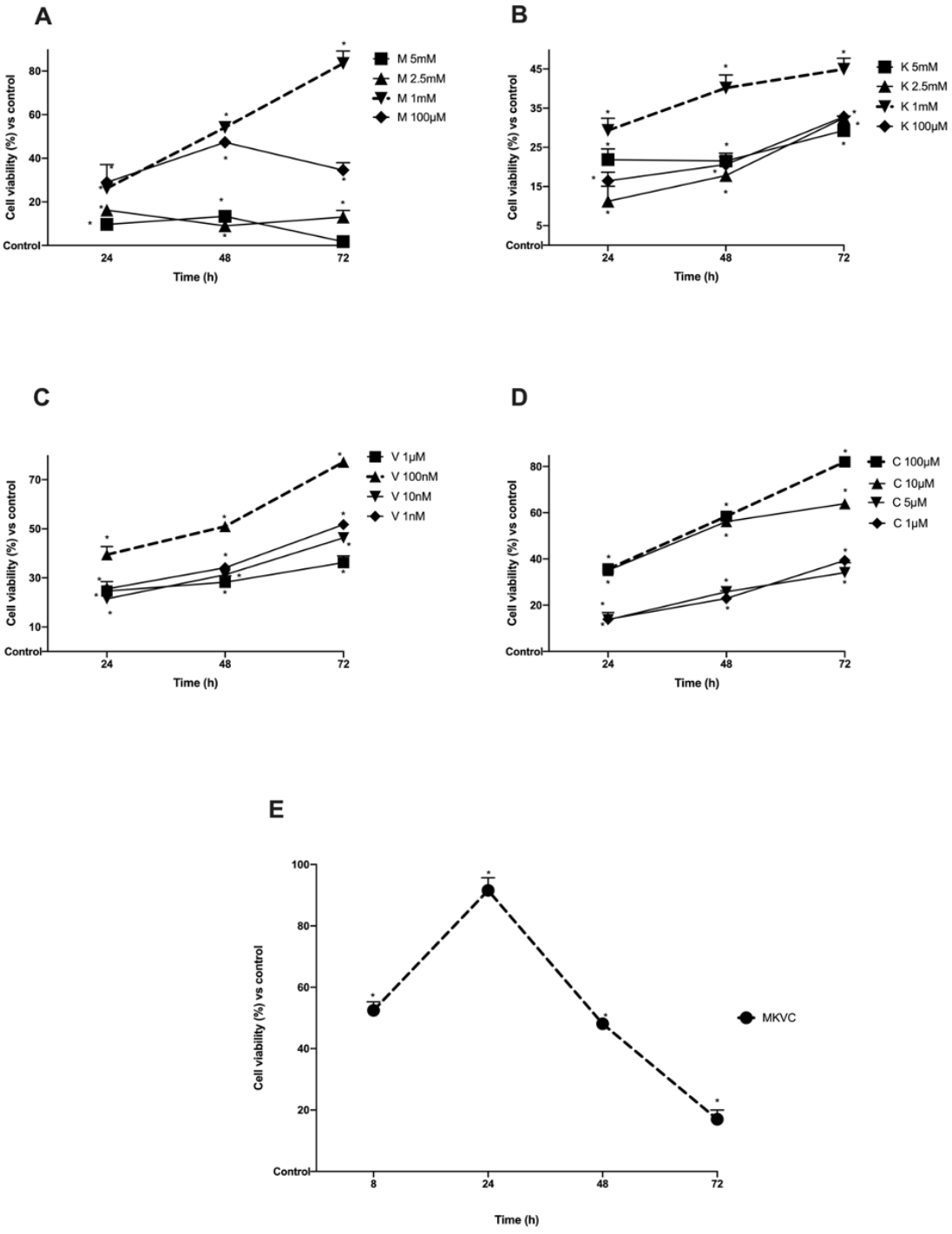

Figure 1. cell viability measured by MTT. From panel **A** to **D**, single agents were tested alone in a dose-response and time-dependent study. M=magnesium in panel a; K=potassium in panel b; V=vitamin D3 in panel c; C=curcumin in panel d. In **E** the cell viability of the combined product named MKVC of effective selected dose of each single agent. Data reported are expressed as means ± SD of five independent experiments. *$p < 0.05$ vs control.

As shown in Fig. 1A-D, in the first set of experiments all substances were able to induce an improvement in cell viability during time without cytotoxic effects. Particularly, M and K (Fig.1A and B, respectively) showed an inversely proportional effect where the 1 mM concentration was able to induce a greater effect than the other concentrations throughout the analyzed period ($p < 0.05$); while V and C (Fig. 1C and D, respectively) showed a directly proportional effect to the used dose. V 100 nM and C 100 µM were able to induce greater effects than other concentrations tested throughout the analyzed period. All these data confirmed that none of the substances had cytotoxic effects overtime on muscle cells; 1 mM for M, 1 mM for K, 100 nM for V and 100 mM for C were the concentrations tested in combination and maintained in all subsequent experiments. As reported in Fig. 1E, the effect of the combination of tested substances, named MKVC, was also investigated on cell viability to verify its effectiveness and to exclude any negative effects. Data confirmed a time-dependent effect with a maximum effect at 24 h compared to control ($p < 0.05$, about 92%) and to all other time points ($p < 0.05$, about 52% at 8 h, 48% at 8 h and 17% at 72 h), indicating the effectiveness of the combination in stabilizing the effects of individual agents on mitochondrial health over time.

Morpho-functional changes in C2C12 cells treated with MKVC

To understand if this new combination was also able to induce a physiological differentiation on myotubes of C2C12 cells, further experiments were carried out by stimulating the cells for 24 h and 72 h by analyzing the differentiation phases, using cyclin D1 and Desmin by Western blot analysis (Fig. 2A and B, respectively).

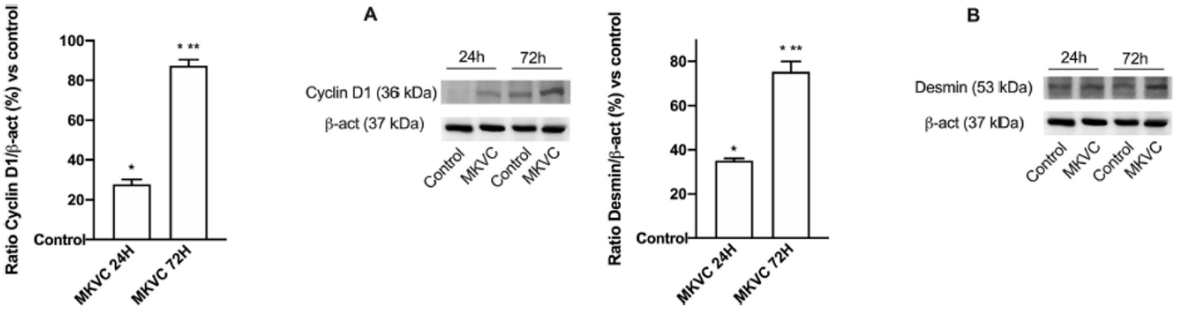

Figure 2. western blot and densitometric analysis. In **A** cyclin D1 and in **B** Desmin expressions caused by MKVC at 2 time points (at 24 h and 72h) and are normalized through β-actin. Data are reported as means ± SD of six independent experiments. *$p < 0.05$ vs control; **$p < 0.05$ vs 24 h stimulation. The abbreviations are the same asreported in Fig.1.

The MKVC combination was able to induce a greater effect than the control ($p < 0.05$) confirming that cyclin D1 expression is abundant in proliferating myoblasts and similar data were derived from Desmin analysis. Furthermore, this combination induced a greater effect at 72 h than at 24 h ($p < 0.05$ vs control) indicating that differentiation began at 24 h (about 27.7% and 35% on both proteins, respectively) confirming the hypothesis of better effect in conditions of intense activity.

Mitochondrial activity is crucial for myoblast proliferation, and it is always accompanied by increased ATP production and oxygen consumption.

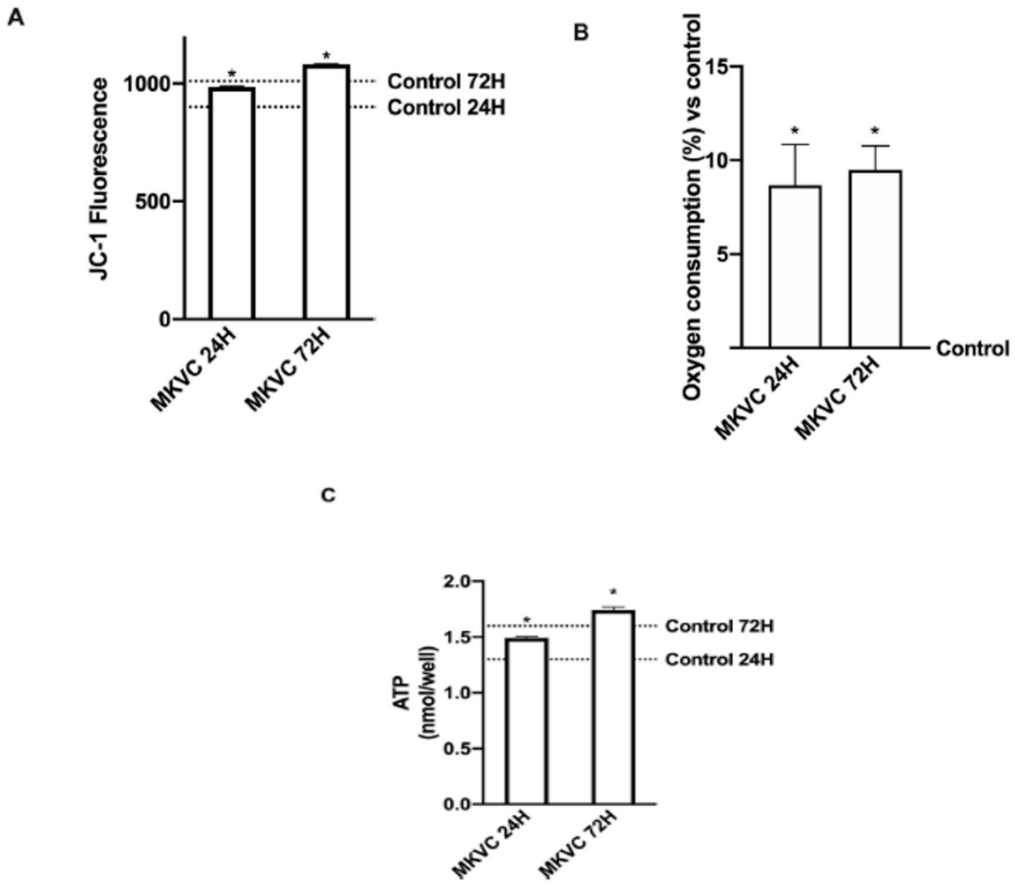

Figure 3. analysis of cellular energy. In **A** mitochondrial potential membrane, in **B** oxygen consumption and in **C** ATP levels caused by MKVC at 2 time points (24 h and 72h). data are reported as means ± SD of six independentexperiments. *$p < 0.05$ vs control. The abbreviations are the same as reported in Fig.1

The analysis of mitochondria by JC-1 suggested that MKVC has a significant impact on mitochondrial activity, particularly on mitochondrial membrane potential. It was able to induce a greater increase compared to control ($p < 0.05$) at 24 h versus 72 h (about 9% and about 7%, respectively), as shown in Fig. 3A, confirming a physiological increase in the chemical energy of the cells.

For more information on mitochondrial metabolism during exercise, oxygen consumption and ATP production were also studied. As shown in Fig. 3B and C, the rate of oxygen consumption and ATP production played an important role in myoblast proliferation.

Indeed, MKVC induced an increase in oxygen and ATP consumption compared to the control ($p < 0.05$) and between the two times at 24 h, no significant changes were observed that could indicate the physiological activity of the mitochondria.

Calcium-magnesium flux analysis to determine the effects on the biology of contraction-relaxation cycle

As it is known, muscle contraction during exercise depends on variations in the intracellular concentrations of calcium ions (Ca^{2+}). Since the behavior of calcium in C2C12 cells is like that observed in *in vivo* experiments, changes in calcium and magnesium levels by Fura-2AM and Furaptra were analyzed *in vitro*, respectively. These experiments were carried out to clarify the importance of Mg^{2+} and Ca^{2+} movements after treatments with MKVC under free-Mg^{2+} and Ca^{2+} medium conditions.

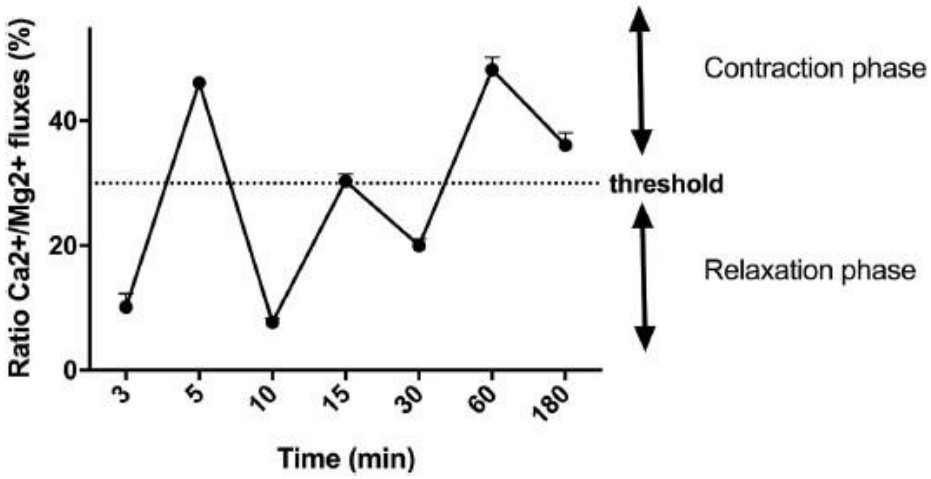

Figure 4. effect of MKVC on muscle contraction. The graph shows a ratio from calcium movements and magnesium flux normalized to control values ranging from 3 min to 180 min. After MKVC administration (time 0), a series of oscillations of the Ca^{2+}/Mg^{2+} ratio was observed which correspond to cycles of contraction and relaxation of muscle cells. Data are reported as means ± SD (%) of five independent experiments. All time points are *p <0.05 vs control.

The balance between calcium and magnesium movements is a reference of the cycles of contraction and relaxation and, as reported in Fig. 4, it showed an alternating pattern from 3 min to 180 min with respect to the control ($p > 0.05$). The presence of magnesium in the formulation produces a physiological movement of calcium ions. This up and down flow was

supposed to be related to the contraction-relaxation cycle. MKVC showed a significant effect over time that was significant at 5 min and 60 min (45% and 47% respectively), supporting MKVC effects on the contraction/relaxation cycle. In addition, these effects regulate a physiological balance between Mg^{2+} and Ca^{2+} flux. Data suggest that MKVC can reduce electrolyte flux peaks also at a physiological level, indicating lower energy consumption during muscle activity over time.

Assessment of muscle activity and inflammation in C2C12 cells

Following the treatment, an immune response could be triggered to assist myoblast proliferation. Since TNFα expression can increase myoblast proliferation, to understand if pro-inflammatory cytokines play a role in this context, TNFα quantification was performed on C1C12 myoblast at the two-time point chosen on the effects on cell viability at 8 h (the beginning of effect) and 24 h (maximum effect).

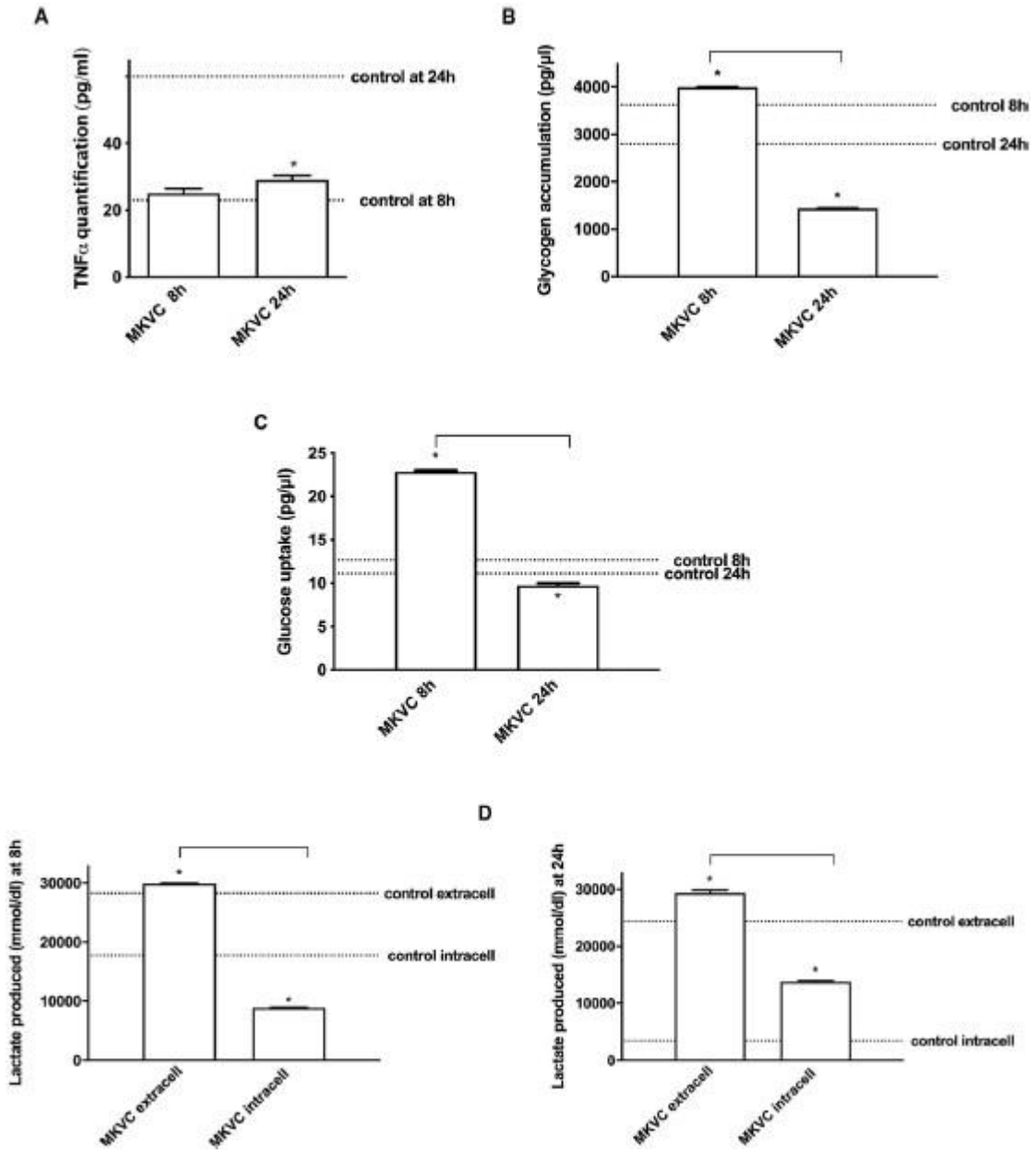

Figure 5. Analysis of several contraction parameters. In **A** TNF-α ELISA assay, in **B** glycogen accumulation, in **C** glucose uptake, and in D lactate analysis with intracellular and extracellular quantification. All these parameters were investigated at two time points. Data are reported as means ± SD of five independent experiments. *$p < 0.05$ vs control; the bars $p < 0.05$ between the 2 time points. The abbreviations are the same as reported in Fig 1.

As shown in Fig. 5A, MKVC induced TNFα release at 8 h hours and 24 h (about 8.7% respect with control and 16% respect with 8 h) in a physiological way; indeed at 8 h the production was near the control values ($p > 0.05$) and at 24 h the production was less than control ($p < 0.05$) indicating that no inflammation response was taking place. Furthermore, since MKVC administration results in normal cytokine production under physiological conditions over time, the data suggest that this formulation may be more effective in hypercontractility conditions.

Since muscle is the most important user of glucose, the accumulation and degradation of glycogen in skeletal muscle plays a central role in systemic glucose homeostasis. To evaluate glucose/lactate concentration and glycogen accumulation, C2C12 cells were treated with MKVC, and their respective analyses were performed. As shown in Fig. 5B and C, glycogen and glucose concentrations were more present in the myoblast at 8 h than 24 h compared to control ($p < 0.05$) (about 10.5% and 79% respectively). The glucose concentration showed the same trend as the glycogen variation suggesting that these two main reserves of muscle fuels were consumed under physiological conditions after MKVC stimulation. At the base of this mechanism there is also the lactate role, which is a metabolic intermediate mainly produced in muscles under anaerobic conditions, especially during exercise. Lactate was previously regarded as "a metabolic waste product" but is now known to be an important fuel source, either used within cells or exported to adjacent organs. Lactate analysis was performed by intra- and extra-cellular analysis and as shown in Fig. 5D, the extracellular level was more abundant at 8 h than 24 h with respect to control ($p < 0.05$); at 8 h lactate level was about 2% with respect to 24 h. These data suggest that under physiological conditions, MKVC induces a lower intracellular accumulation of lactate with consequent greater consumption of glucose supporting the aerobic activity.

Analysis of the intracellular pathways activated by MKVC on C2C12 cells

To explore whether MKVC can support different phases and moments in the intracellular mechanisms involved in an exercise, further experiments were performed by analyzing some important kinases in muscle cells.

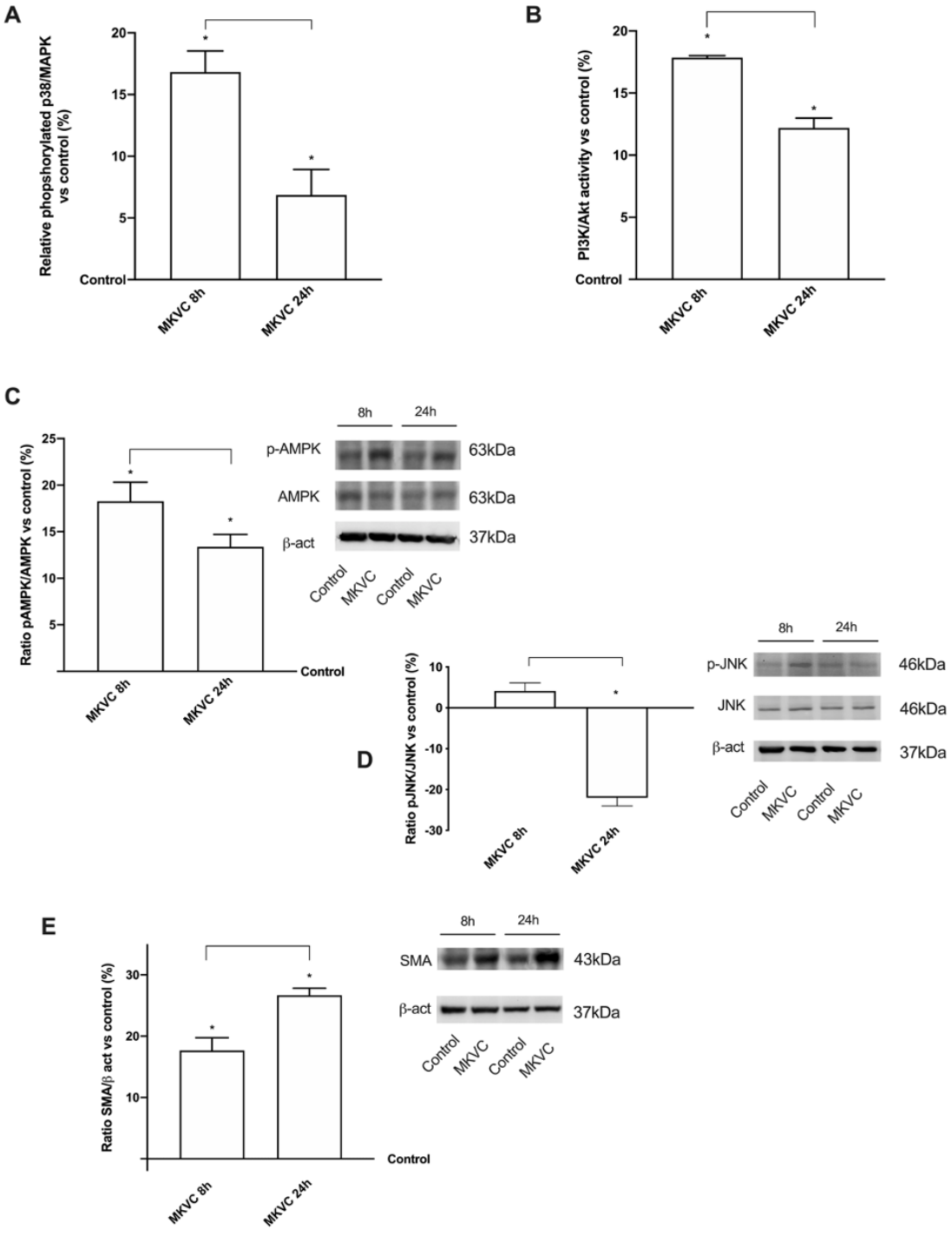

Figure 6. Protein activities, Western blot, and densitometric analysis in C2C12 cells. In **A** p38 phosphorylation and in **B** PI3K/Akt activity both measured by ELISA test. Data are reported as means ± SD of four independent experiments reported as % vs control (0 line). *p <0.05 vs control; the bars between 8h and 24 h of MKVC stimulation. In **C** AMPK phosphorylation, in **D** JNK phosphorylation and in **E** SMA expression were analyzed by western blot and densitometric. The images represent the expression of each protein from five independent experiments normalized on specific total protein when possible and verified by β-actin detection. Data are reported as means ± SD (%) vs control (0 line).

As reported in Fig. 6A, the activity assay of p38/MAPK was analyzed as a marker of contractile response at 8 h and 24 h of stimulations. Data confirmed MKVC's role in aiding the activation of this intracellular pathway at 8 h. Moreover, the effect at 24 h is reduced leading to the energy conservation state. In contrast, the effect at 8 h supported previous findings on the proliferation phase induced by MKVC compared to control ($p < 0.05$). A second major pathway supporting protein synthesis and glucose uptake, PI3K/Akt (Fig. 6B), confirmed its ability to support a late phase of cell activation since MKVC can induce its activation ($p < 0.05$) compared to control. Furthermore, at 24 h its activation was reduced compared to 8 h ($p < 0.05$, about 46%) but it was increased compared to control ($p < 0.05$, about 12%) indicating that the effects on myoblasts were maintained over time.

Another key regulator of cellular metabolism, which plays a predominant role in catabolic mechanisms is the glucose transporter AMPK, which has also been studied. As reported in Fig. 6C MKVC appears to improve muscle metabolism at 8 h in which a greater expression of AMPK is observed (about 36% than 24 h, $p < 0.05$) compared to control ($p < 0.05$, about 18%). The effectiveness of MKVC is maintained over time and demonstrated for the first time that MKVC can induce glucose uptake according to the classic activation mechanisms with mitochondrial modulation. Furthermore, to support the effectiveness of the contraction and to rule out the inflammatory response, the evaluation of JNK 1/2 was carried out. As reported in Fig. 6D, MKVC improved the biology linked to contractile response, offering a physiological picture of the muscular cell activity at 8 h, it maintained an effect equal to the control which was drastically reduced at 24 h supporting a lower level of hypercontraction with a reduction of 5.5 times in the presence of high glucose. These data support the effectiveness of MKVC in addition to improving metabolism and in reducing any associated inflammatory processes.

Finally, other experiments were conducted evaluating smooth muscle actin (SMA) as a marker of muscle regeneration, including post-trauma and anti-muscle damages. As demonstrated in Fig. 6E, after MKVC stimulation, myoblasts appear better at maintaining physiological contractile activities at 8 h as they had a greater effect compared to control ($p < 0.05$ about 17.7%), and this effect was amplified at 24 h compared to control ($p < 0.05$) and to 8 h ($p < 0.05$ about 50.8%), indicating that this effect is consolidated over time. These data support the *in vitro* efficacy of MKVC in maintaining muscle survival systems, even after hypercontraction-related damage.

Simulated hypercontraction induced by caffeine

It is well established from *in vitro* studies that caffeine has a direct effect on muscle contraction. It plays a key role in the functioning of the sarcoplasmic reticulum, increasing calcium permeability and making it readily available for the contraction mechanism. Caffeine was administered to the C2C12 cells to provide a treatment that simulates strenuous exercise, thus studying the biological aspects of exercise to examine exercise-regulated changes in signal transduction and metabolism. Cells were pre-treated with 2.5 mM caffeine and then treated with MKVC at 8 and 24 h. To understand the effects on contraction-related molecular mechanisms, calcium, and magnesium levels were analyzed.

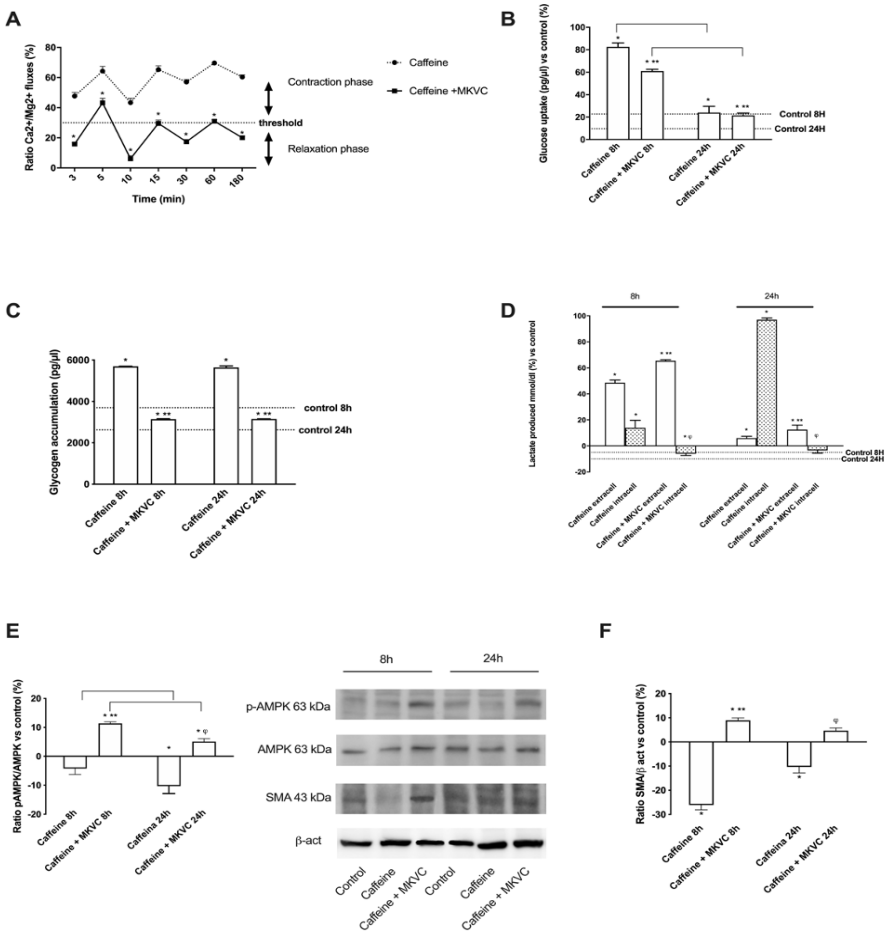

Figure 7. Effects of caffeine on C2C12 cells. In **A** the graph shows a ration from calcium movements and magnesium flux normalized to control values ranging from 3 min to 180 min. After the administration (time 0), a series of oscillations of the Ca^{2+}/Mg^{2+} ratio was observed, which correspond to cycles of contraction and relaxation of the cells. Data are reported as means ± SD (%) of five independent experiments. In **B** glucose uptake, in **C** glycogen accumulation, and in **D** lactate analysis with intracellular and extracellular quantification. All these parameters were investigated at two time points (8h and 24 h). data are reported as means ± SD of five independentexperiments. *p < 0.05 vs control; ***p < 0.05 vs caffeine; $^{\Psi}$ p < 0.05 vs intracellular caffeine; the bars p < 0.05 the same treatment between the different time points. In **E** AMPK phosphorylation and in **F** SMA expression were analyzed by western blot and densitometric analysis. The images represent the expression of each protein from five independent experiments normalized on specific total protein when possible and verified by β-actin detection.Data are reported as means ± SD (%) vs control (0 line). *p < 0.05 vs control; ** p < 0.05 vs caffeine 8h; $^{\Psi}$ p <
0.05 vs caffeine 24 h.

As reported in Fig. 7A, caffeine does not allow an adequate relaxation phase. On the other hand, MKVC has a better contraction modulation effect (about 15% less than caffeine), supporting the effects of MKVC in facilitating relaxation after hypercontraction, compared to control (p < 0.05) and compared to caffeine treatment, which instead delays relaxation (p < 0.05). Ultimately, these data state that MKVC better modulates calcium movements, bringing

them back to more physiological values and remodeling the contractile phase with less cellular fatigue.

Since *in vivo* exercise induces a cascade of intracellular mechanism (e.g., activation of AMPK), proteomic (e.g., GLUT4), and metabolic changes (e.g., increased glucose uptake), additional experiments *in vitro* were carried out to confirm the effects of MKVC.

Consequently, additional parameters such as glucose uptake, glycogen consumption, and lactate accumulation were studied following pre-treatment with caffeine. As shown in Fig. 7B-D, caffeine alone induced a biological response compatible with a state of severe contraction accompanied by high glucose uptake, high glycogen concentration, and a significant increase in extracellular lactate ($p < 0.05$). This indicates a negative condition of the cells compared to a state of hypercontractility. The stimulation with MKVC was able to reduce this negative condition, leading the extracellular lactate to the control value ($p < 0.05$ vs caffeine alone).

Since the strong contraction can cause muscle hypertrophy accompanied by cell death in which the stress condition is maintaining for a long time, additional experiments were performed analyzing some intracellular pathways leading to glucose transport (e.g., AMPK) and muscle regeneration (e.g., SMA). As reported in Fig. 7E, following stimulation with caffeine, MKVC allows the restoration of normal glucose uptake with greater effectiveness at 8 h, compared to control ($p < 0.05$). MKVC was able to modulate the AMPK pathway after caffeine stimulation suggesting its ability to restore and recover muscle cell metabolism after hypercontractility. Finally, the analysis of regeneration and myogenesis markers such as SMA showed similar results (Fig. 7F). Indeed, MKVC was able to counteract the negative effect of caffeine and the main effects were observed at 8 h compared to control ($p < 0.05$) and compared to caffeine about twice. In addition, this effect is maintained over time suggesting a long-term effect on the restoration of functionality and on complete post-trauma regeneration.

All reported data are published in: "*Molinari C, Ruga S, Farghali M, Galla R, Bassiouny A, Uberti F. Preventing c2c12 muscular cells damage combining magnesium and potassium with vitamin D3 and curcumin. J Tradit Complement Med. 2021 Jun 2;11(6):532-544. doi: 10.1016/j.jtcme.2021.05.003. PMID: 34765517; PMCID: PMC8572722.*"

4.2. Project 2: Retinae Aging

The obtained results demonstrate the right maintenance of retinal trophism, thanks to Gastrodin and Vitamin D which help to prevent oxidative stress damage and inflammation.

Analysis of cell viability and ROS production in RGC cells treated for 8 days

The MTT test was performed in a dose-response study to identify the best concentration of gastrodin (G) to be used in all subsequent experiments on RGC cells. A concentration in the range of 1 to 100 µM was chosen for 8 days.

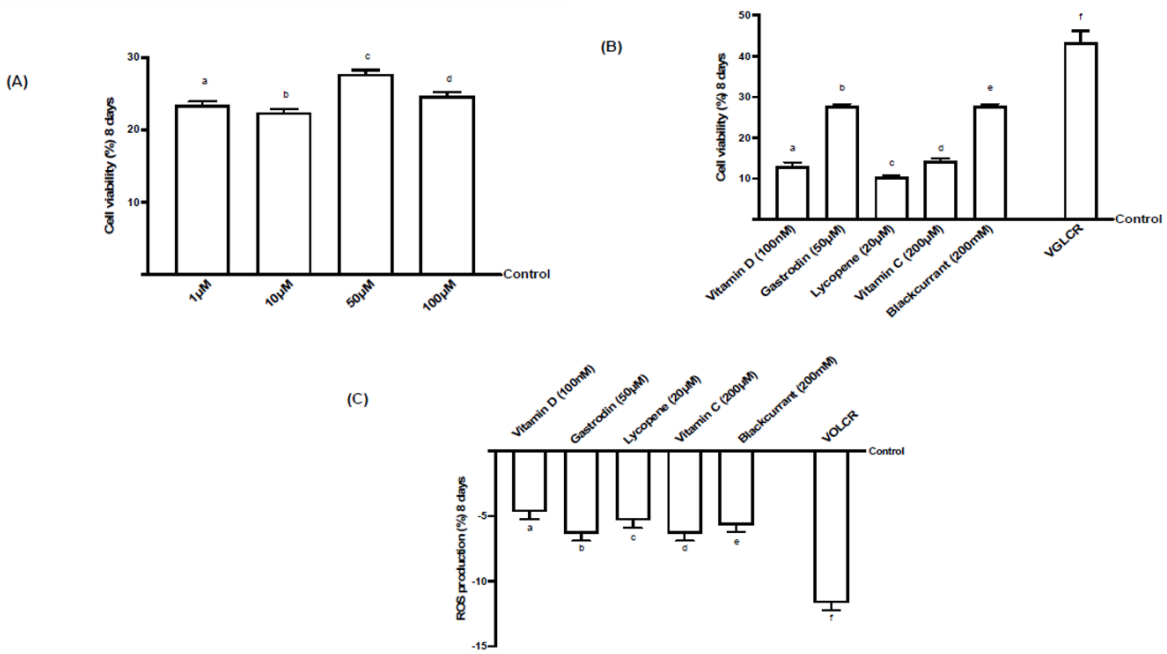

Figure 8. Cell viability and ROS production on RGC cells. In (**A**), dose-response study after gastrodin administration (1µM-100 µM) on cell viability. Results are expressed as means ± SD (%) vs control (0% line) of 4 independent experiments each performed in triplicates. a, b, c and d are p <0.05 vs control; c is p <0.05 vs a, b and d. In (**B**), the effects of V=vitamin D, G=gastrodin, L=lycopene, C=vitamin C, R=blackcurrant, and VGLCR combination on cell viability. In (**C**), the effects of V=vitamin D, G=gastrodin, L=lycopene, C=vitamin C, R=blackcurrant, and VGLCR combination on ROS production. Both (**B**, **C**) results are expressed as means ± SD (%) vs control (0% line) of 4 independent experiments, each performed in triplicates. a, b, c, d, e and f p<0.05 vs control; f is p<0.05 vs a, b, c,d and e.

As reported in Figure 8A, G was able to induce a significant increase ($p < 0.05$) in cell viability compared to the control. Particularly, 50 µM of this substance induced the best significant increase ($p < 0.05$), compared to the other concentrations tested (about 18.9% with 1 µM; about 24.2% with 10 µM; and about 12.3% with 100 µM). For this reason, G 50 µM was maintained for all subsequent experiments.

Additional experiments were carried out to exclude any cytotoxic effect induced by vitamin D3 (V, 100 nM), gastrodin (G, 50 µM), lycopene (L, 15 µM), vitamin C (C, 200 µM), and blackcurrant (R, 200 µM) alone and together, by analyzing cell viability after 8 days of stimulation on RGC cells. As shown in Figure 8B, all these substances tested alone were able to induce a significant increase in mitochondrial metabolism ($p < 0.05$) compared to the control. This provides the scientific basis for hypothesizing a new formulation, based on the combination of these substances. In addition, the combination of V 100 nM, G 50 µM, L 15 µM, C 200 µM, and R 200 µM, named VGLCR, confirmed this hypothesis. After 8 days of treatment, VGLCR was able to amplify ($p < 0.05$) the beneficial effect observed using single compounds. VGLCR had effects greater than about 50% compared to R and G alone, about three times compared to C, about four times compared to L, and three times compared to V. In particular, the results on cell viability confirmed that the greatest effects were obtained after 8 days of stimulation for all treatments, and VGLCR confirmed to have a better action throughout the analyzed period compared to all the other substances ($p < 0.05$). These data confirmed the absence of adverse effects during the treatment and the effectiveness of the combination, suggesting a synergistic effect exerted by the sum of all compounds. Furthermore, since reactive oxygen species (ROS) have been implicated in the pathogenesis of various eye diseases, including mitochondrial dysfunction, ROS production after 8 days of stimulation with all compounds alone or combined was investigated. As shown in Figure 8C, the antioxidant property of V 100 nM, G 50 µM, L 15 µM, C 200 µM, and R 200 µM alone was confirmed, and VGLCR produced an amplified effect ($p < 0.05$) with respect to individual agents and control. All these data confirm the effectiveness of VGLCR on mitochondrial balance, supporting the hypothesis that it can be used to prevent retinal cell loss. For this reason, only the VGLCR combination was tested in all subsequent experiments.

Cell viability and ROS production in RGC cells subjected to conditions mimicking Glaucoma

Since VGLCR may be used both before and during the treatment of glaucoma, some experiments on RGC cells were performed before and after the induction of cell damage, mimicking glaucoma by means of NMDA and H_2O_2. These agents are classical glaucoma inductors capable of mimicking the damage caused by high ocular pressure and oxidative damage, and for this reason they have been added alone or together for 8 days.

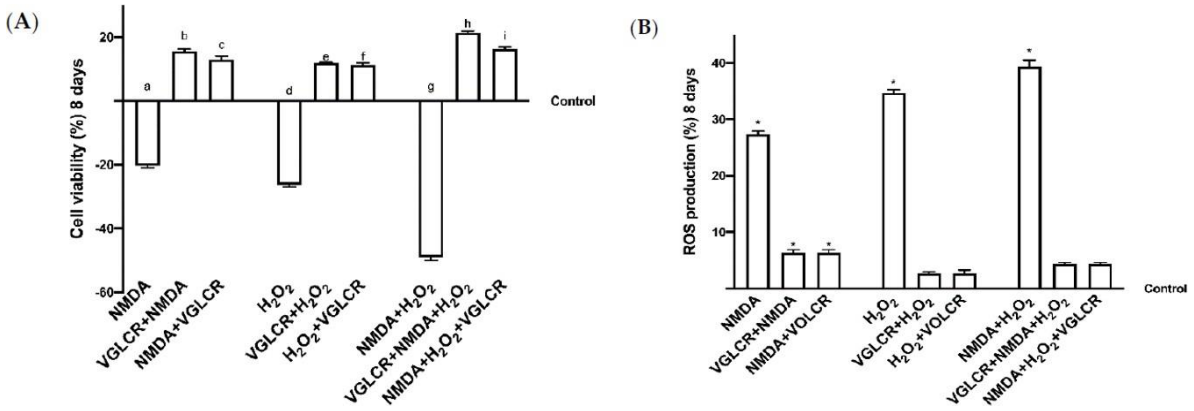

Figure 9. Cell viability and ROS production of RGC cells treated with NMDA and H_2O_2. In (A), cell viability is measured after NMDA and H_2O_2 alone and together before and after VGLCR stimulation. Results are expressed as means ± SD (%) vs control (0% line) of 4 independent experiments, each performed in triplicates. a-i $p < 0.05$ vs control; b and c $p < 0.05$ vs a; c $p < 0.05$ vs b; e and f $p < 0.05$ vs d; I and h $p < 0.05$ vs g; I $p < 0.05$ vs h. in (B), the same stimulation used in panel (A) to analyze ROS production. Results are expressed as means ± SD (%) vs control (0% line) of 4 independent experiments each performed in triplicates. * $p < 0.05$ vs control. Abbreviations are the same as reported in Figure 8.

As shown in Figure 9A, NMDA and H_2O_2 alone confirmed their role as glaucoma inductors, reducing cell viability ($p < 0.05$ vs. control) and indicating the presence of an injury to RGC cells. The simultaneous treatment with NMDA and H_2O_2 after 8 days amplified this reduction in cell viability ($p < 0.05$) compared to NMDA and H_2O_2 alone (about 69% and 88%, respectively). This stimulation indicates a severe damage of RGC cells, like the human glaucomatous condition. To demonstrate the effectiveness of VGLCR as a therapeutic adjuvant, both during the early stage of this condition and during the overt disease the stimulation was performed before and after NMDA and H_2O_2 alone and together. In all conditions tested, VGLCR was able to restore the induced damage, confirming a positive role on cell viability compared to control and glaucoma inductors ($p < 0.05$). In particular, the main effects were observed when NMDA and H_2O_2 were added together, proving the existence of a

beneficial effect of VGLCR against a glaucomatous condition. At the same time, VGLCR appears to have more of an effect ($p < 0.05$) when added prior to glaucoma induction, using NMDA and H_2O_2 together, indicating a possible role in the early stage of the disease. Similarly, the analysis of ROS production, shown in Figure 9B, confirmed the negative effects of NMDA and H_2O_2 alone and together ($p < 0.05$ vs. control). In particular, significant damage ($p < 0.05$) was obtained by summing together the glaucoma inductors. VGLCR was able to prevent and restore all these negative conditions, confirming what was observed in cell viability. Since the main negative effect was obtained with the administration of NMDA+H_2O_2, only this treatment was used to induce the damage on all subsequent experiments.

Analysis of the main intracellular pathways activated in RGC subjected to conditions mimicking Glaucoma

Since previous findings have demonstrated the ability of VGLCR to induce beneficial effects on retinal cells, it was important to verify the intracellular mechanisms leading to these effects. This involved analyzing the activation *in vitro* of main pathways linked to glaucoma.

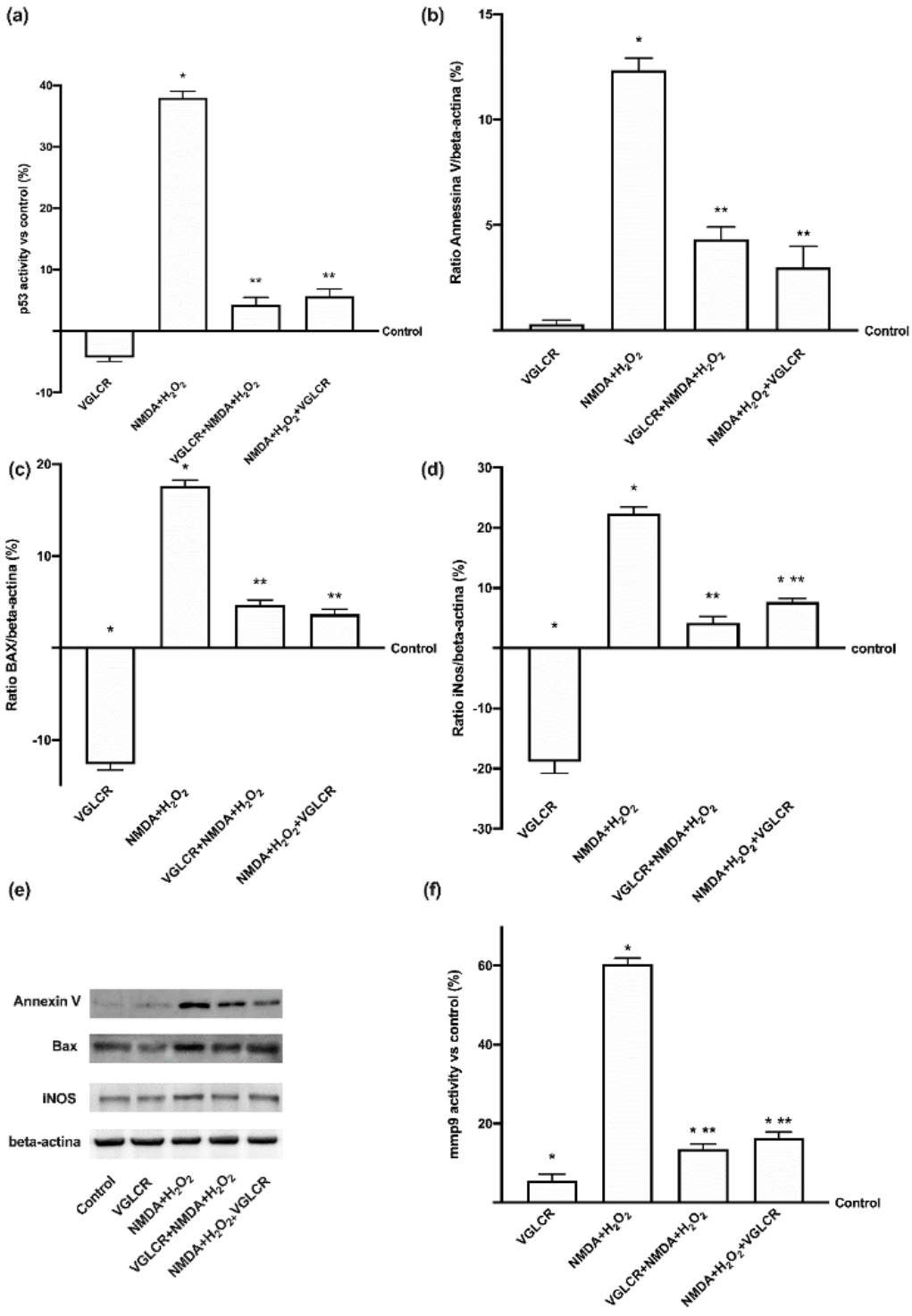

Figure 10. Western blot, densitometric analysis, and ELISA activity of main intracellular glaucoma markers. In (**A**) p53 activity measured by ELISA test; in (**B**) densitometric analysis of Annexin V; in (**C**) densitometric analysis of Bax; in (**D**) densitometric analysis of iNOS; in (**E**) western blot images of Annexin V, Bax, and iNOS; (**F**) MMP9 activity measured by ELISA test. All these markers were measured with NMDA+H_2O_2 before or after VGLCR stimulations. Results are expressed as means ± SD (%) vs control (0% line) of 4 independent experiments each performed in triplicates. Densitometric analyses are expressed as means ± SD (%) of 4 independent experiments, normalized and verified by β-actin detection. Images shown are an example of each protein from 4 independent experiments. * $p < 0.05$ vs control; ** $p < 0.05$ vs NMDA+H_2O_2

As reported in Figure 10, the effects of VGLCR before and after the administration of NMDA+H_2O_2 on the activity of p53 and on the expression of Annexin V and Bax were investigated. The glaucoma inductors added together confirmed their negative effects on RGC cells, and the subsequent cell loss is reported in Figure 10A for p53 activity compared to the control ($p < 0.05$). Conversely, VGLCR alone did not activate p53 ($p > 0.05$ vs. control), but if added before or after the glaucoma inducers it was able to counteract the adverse effects ($p < 0.05$ vs. NMDA+H_2O_2, approximately eight times and seven times greater in both conditions, respectively). In particular, the combination appears to be able to induce a greater effect if added before rather than after the induction of damage (about 30%), but this difference is not statistically significant, revealing a limitation of these *in vitro* experiments. In addition, this beneficial effect was also confirmed by analysis of Annexin V and Bax, as reported in Figure 10B, C, E. NMDA+H_2O_2 induced a significant increase in Annexin V expression compared to the control ($p < 0.05$), supporting data on the nuclear compartment damage of RGC cells that can cause cell death, as suggested by the observations in cell viability, ROS analysis, and p53 activity. Conversely, VGLCR added before or after the injury was able to reduce this expression by approximately four times and approximately three times, respectively ($p < 0.05$), indicating its protective effects on RGC cells. The effects obtained before or after the administration of VGLCR support the hypothesis that this combination has a greater effect if added before the damage (about 38% compared to after), but these data need to be confirmed in a more complex experimental system. Furthermore, the analysis of Bax (Figure 10 C, E) shows a significant increase in its expression ($p < 0.05$) compared to the control in the presence of glaucoma inductors, indicating the presence of RGC cell apoptosis and confirming that the association of NMDA+H_2O_2 mimicked the real negative consequences of glaucoma in humans. VGLCR was able to counteract these negative effects if added both before and after the injury, reducing ($p < 0.05$) the expression of Bax compared to that which happens after the inductors (about five times if added before and four times if added after, respectively). Among the effects obtained by adding VGLCR either before or after, the results confirmed the hypothesis that it has a great effect if added before the damage (about 27% compared to after), but this data need

to be confirmed. These findings demonstrated for the first time that NMDA and H_2O_2 combined reproduced an *in vitro* glaucomatous condition, and that VGLCR was able to prevent RGC cell loss.

At this point, since oxidative stress is involved in cell apoptosis, an analysis of iNOS was performed to confirm the role of its modulation in preventing cell death. As illustrated in Figure 10D, E, NMDA+H_2O_2 induced a significant increase in iNOS expression ($p < 0.05$) compared to the control, supporting previous data on ROS production, p53 activity, and both Annexin V and Bax expressions. Conversely, stimulation with VGLCR before and after damage was able to reverse this negative condition (about five times if added before, and three times if added after). This supports the hypothesis that the antioxidant activity is maintained by VGLCR, and this counteracts oxidative stress. In particular, the best effect appears to occur when VGLCR is administered before damage (approximately 81% compared to after), but this needs to be confirmed. Among the negative consequences, the alteration of the activity of MMPs seems to be very important, leading to an altered composition of the extracellular matrix, which involves MMP9-activity (Figure 10F). NMDA+H_2O_2 confirmed the ability to induce the damage by activating MPP9 ($p < 0.05$ vs. control), leading to cells loss; VGLCR was able to significantly decrease the MPP9 activation when added both before (about 4.4 times compared to damage) and after the injury (about 4 times compared to damage), confirming its ability to improve cell viability by modulating oxidative stress and apoptosis of RGC cells. Pre-treatment with VGLCR confirmed an approximately 19% better effect than post-stimulation, but further experiments may be needed to fully clarify this observation. All these findings demonstrated for the first time that VGLCR can reverse cell degeneration and prevent cell death, by modulating the intracellular pathways involved in human glaucoma. Some additional experiments were carried out to evaluate the activity of MAPK/kinases. Particularly, ERKs/MAPK, p38 MAPK, and JNK1/2 activities were studied, as they are commonly involved in the early stage of optic nerve degeneration.

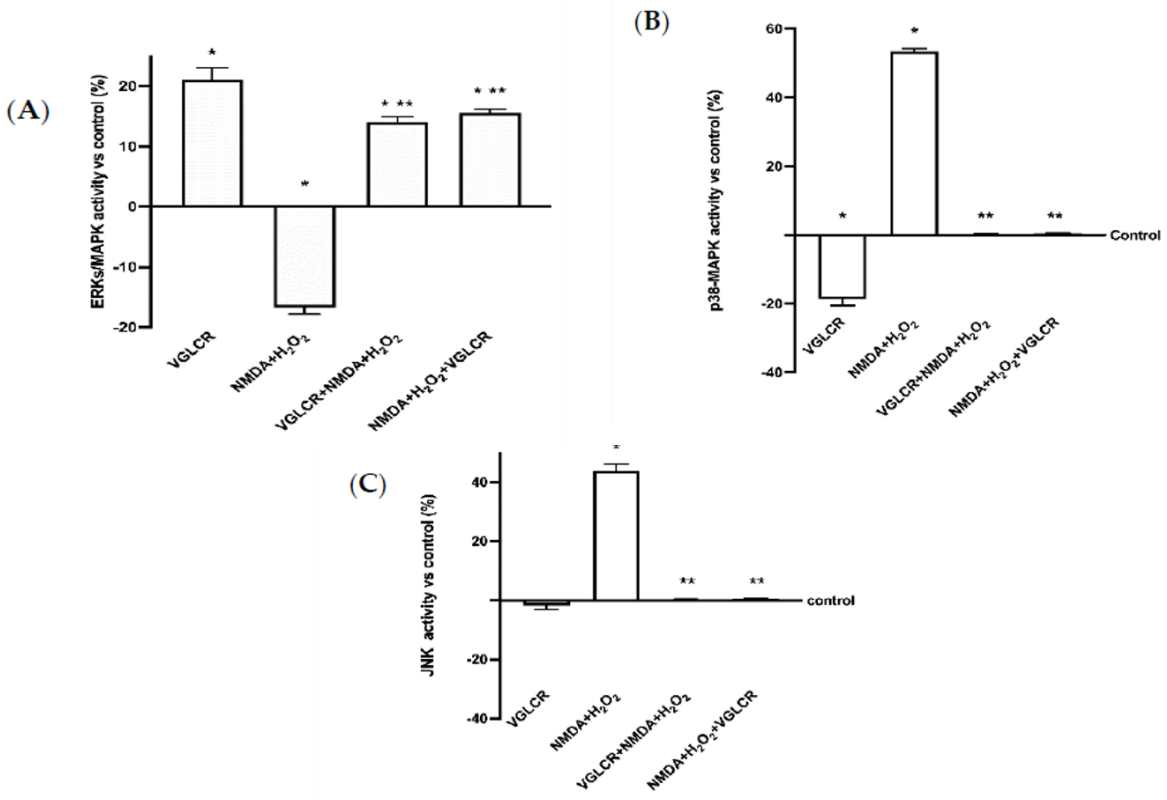

Figure 11. ELISA activity of ERKs (A), p38 (B), and JNK 1/2 (C). All these markers were measured with NMDA and H_2O_2 added together, before and after VGLCR stimulations. Results are expressed as means ± SD (%) vs control (0% line) of 4 independent experiments each performed in triplicates. * $p < 0.05$ vs control; ** $p < 0.05$ vs NMDA+H_2O_2. Abbreviations are the same as reported in Figure 8.

As reported in Figure 11A, the activation of cell survival kinase (ERKs/MAPK) was significantly decreased in the presence of glaucoma inductors, confirming the previously observed negative consequence of the stimulation with NMDA+H_2O_2 ($p < 0.05$ vs. control). On the contrary, the presence of VGLCR, administered both before and after the injury, was able to significantly increase this activity, supporting the survival response of RGC cells ($p < 0.05$ compared to inductors). Furthermore, the effects on p38/MAPK (Figure 11B), a stress kinase, showed similar effects; NMDA+H_2O_2 significantly increased the activity of this cellular stress marker compared to the control ($p < 0.05$), and the presence of VGLCR before and after the damage reverted this negative effect, bringing the activity back to the control values. This confirmed that the inhibition of p38/MAPK activity in the retinal field may represent a strategical therapeutic target for preventing the early stage of pathogenesis in optic neuropathies ($p < 0.05$ vs. NMDA+H_2O_2). Finally, the analysis of JNK1/2 activity (Figure 11C) showed that NMDA+H_2O_2 promoted the degenerative processes by increasing JNK activity ($p < 0.05$ vs. control). On the contrary, the presence of VGLCR before or after the damage reduced

this activation, leading to control values and supporting its ability to prevent and/or restore the injury caused by glaucoma inductors ($p < 0.05$ vs. NMDA+H_2O_2).

Some additional experiments were performed to verify the effectiveness of VGLCR directly administered on complex systems as retinal tissues explants (Figure 12). In these experiments of an *in vitro* experimental model of retinal tissue, the same treatments and conditions used on RGC cells were maintained, analyzing the effects of NMDA+H_2O_2 alone and in the presence of VGLCR added either before or after the experimental injury.

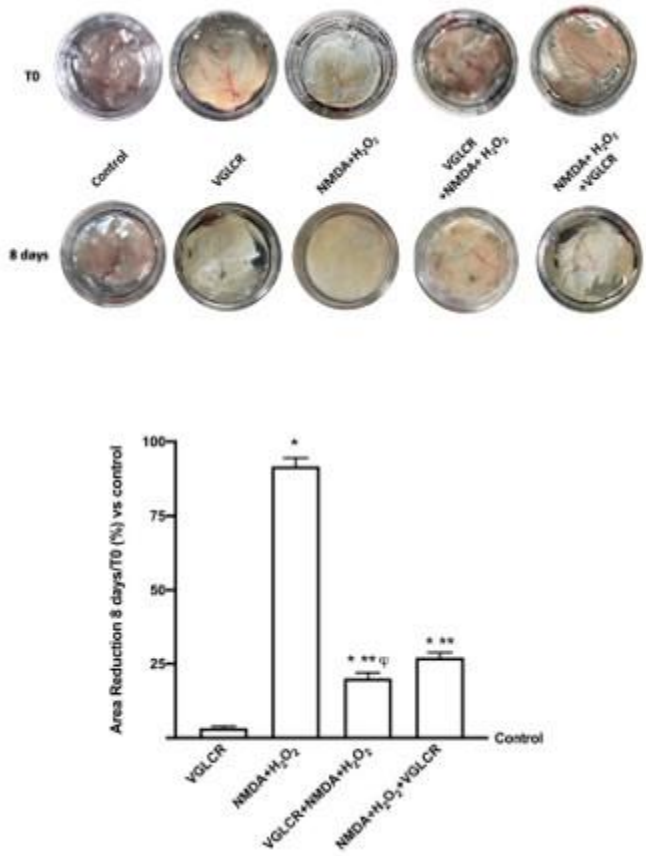

Figure 12. Morphological tissue and relative counting area on retinal tissues treated with NMDA and H_2O_2 added together, before and after VGLCR stimulations. Results are expressed as a reduction area normalized on T0 and the control quantified by ImageJ software, expressed as means ± SD (%) of 4 independent experiments reproduced in duplicates. * $p < 0.05$ vs control; ** $p < 0.05$ vs NMDA+H_2O_2; φ $p < 0.05$ vs NMDA+H_2O_2+VGLCR. Abbreviations are the same as reported in Figure 8.

The macroscopic analysis of the eyecup preparations and the relative measurement of the areas showed that the glaucoma inductors were able to degenerate retinal tissue, thus leading to cell death ($p < 0.05$ compared to control). This observation confirmed the data obtained on RGC cells. In addition, several morphological changes in the tissues treated with VGLCR were

observed compared to the control and to what was observed after administration of NMDA+H_2O_2. Indeed, VGLCR administered before the induction of the glaucomatous lesion was able to prevent the retinal degeneration ($p < 0.05$ compared to glaucoma inductors) better than when the compound was added after the damage (about 35%). At morphological level, in retinal tissues treated with VGLCR, the ophthalmic artery was always visible both with respect to the control and after the stimulation with glaucoma inductors, confirming the hypothesis that VGLCR was able to counteract and/or prevent the negative aspects of glaucoma, making it suitable for supplementing glaucoma therapy.

Beneficial role of VGLCR to prevent/restore the damage caused by NMDA+ H_2O_2 in retinal extracts

Further experiments were carried out directly on the retinal tissue treated as previously described to analyze the effects of VGLCR on the main kinases involved in retinal degeneration and typical of human glaucoma.

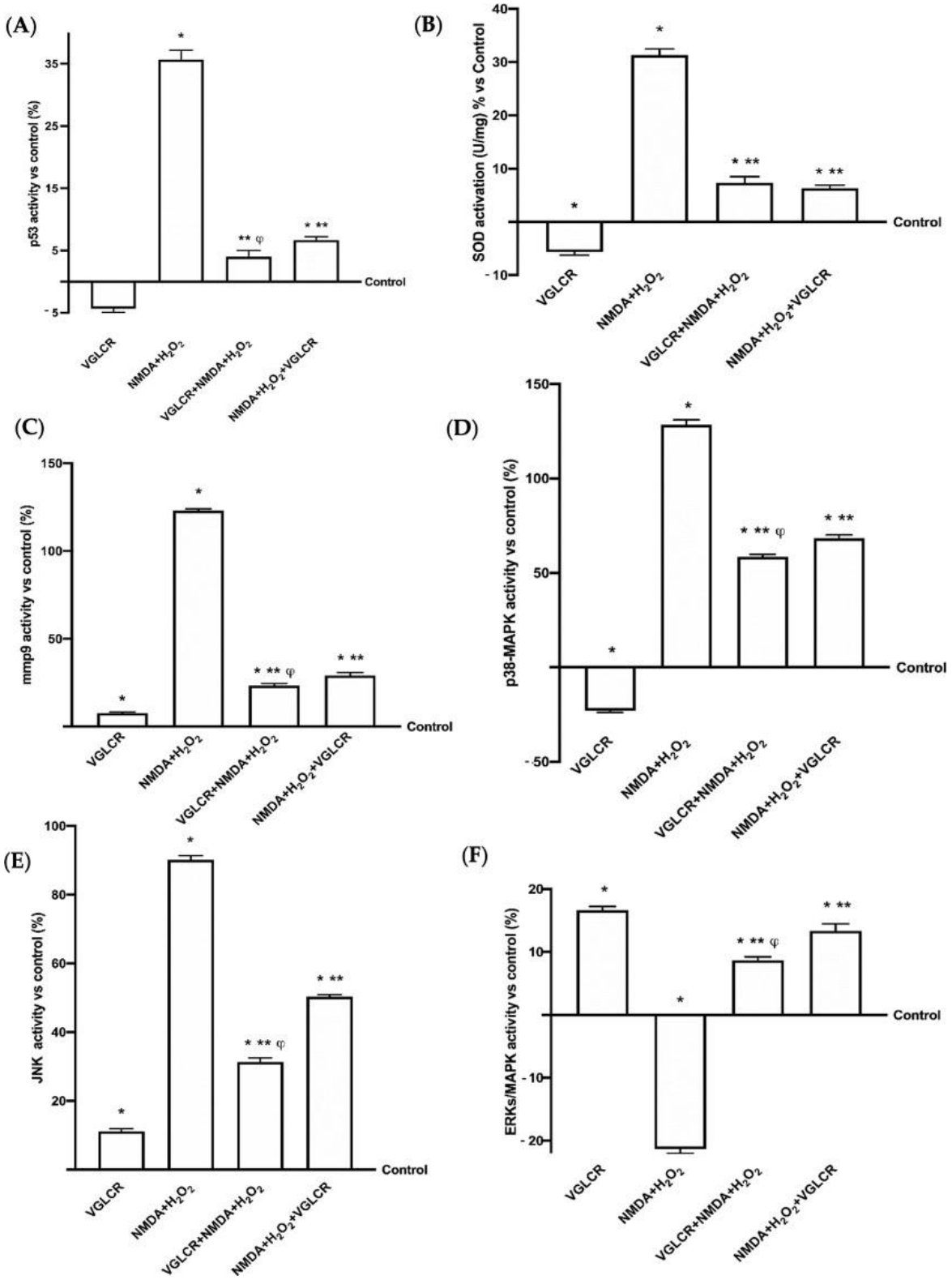

Figure 13. Activity assays of the main markers involved in glaucoma. In (**A**) p53, in (**B**) SOD, in (**C**) MMP9, in (D) p38/MAPK, in (**E**) JNK 1/2, in (**F**) ERKs/MAPK. Retinal tissues were treated with NMDA and H_2O_2 added together, before and after VGLCR stimulations. Results are expressed as means ± SD (%) vs control of 4 independent experiments reproduced in duplicates. * $p < 0.05$ vs control; ** $p < 0.05$ vs NMDA+H_2O_2; φ $p < 0.05$ vs NMDA+H_2O_2+VGLCR. Abbreviations are the same as reported in Figure 8.

As reported in Figure 13A, the level of p53 activity confirmed the loss of retinal integrity, indicating a consequence of administration of NMDA+H_2O_2. VGLCR was able to revert degradation of this tissue by reducing the activation of p53 when added before and after NMDA+H_2O_2 ($p < 0.05$; by about nine times and five times, respectively), confirming its beneficial effects. Analysis of data collected before and after the induction of damage suggested that pre-treatment was better than post-stimulation (approximately 75%, $p < 0.05$), supporting the hypothesis of a potential use as a supplement with preventive action in humans.

Since oxidative stress is considered as a "primum movens" of glaucomatous degeneration, the most important molecular pathways implicated in this critical condition, such as SOD activity (Figure 13B) and iNOS (Figure 14A, E), were also analyzed. Data obtained demonstrated that the SOD level is higher in the presence of NMDA+H_2O_2 ($p < 0.05$ compared to control), and this negative effect was counteracted by the pre-treatment with VGLCR ($p < 0.05$, about 4.5 times), but also significantly reduced by the post-stimulation with the same combination ($p < 0.05$, about five times). Similarly, the analysis of iNOS expression confirmed the negative effects of oxidative stress on retinal tissues treated with NMDA+H_2O_2 ($p < 0.05$ vs. control), and the ability of VGLCR to prevent ($p < 0.05$, about 5.5 times) or restore the damage ($p < 0.05$, about four times). The greater protective action of VGLCR was again confirmed in these experiments; indeed, the pre-treatment induced a greater reduction than the post-stimulation (about 46%, $p < 0.05$). Starting from tissue microscopy observations, in which retinal remodeling and tissue death were observed, additional experiments to investigate MMP9 activity (Figure 13C) and Bax expression (Figure 14B, E) were performed.

NMDA+H_2O_2 has confirmed the degeneration and loss of retinal tissue as reported above ($p < 0.05$ vs. control), and these effects were significantly reduced by the stimulation with VGLCR before ($p<0.05$ about five times and about four times, respectively) and after the injury ($p < 0.05$ about four times and three times). In addition, the pre-treatment with VGLCR exerted a better influence than the post-stimulation ($p < 0.05$ about 26% and 40%, respectively), confirming the findings obtained from experiments on RGC cells and from observations at the macroscopic level. Furthermore, the MAPK pathway implicated in the early stage of glaucoma has been tested on eyecup preparations. As reported in Figure 13 D, E, the p38MAPK and JNK1/2 activities were significantly increased by the presence of NMDA+H_2O_2 ($p < 0.05$ compared to control), confirming the negative consequences of oxidative stress on retinal

tissues. On the other hand, VGLCR added both before and after the damage was able to prevent ($p < 0.05$, about two times and three times, respectively) and/or restore ($p < 0.05$ about 2 times and about 1.5 times, respectively) the glaucomatous condition, confirming its beneficial role to modulate oxidative stress and relative damage at the early stage of glaucoma. Again, in these cases, the main positive modulations on both markers were obtained by adding VGLCR before the induction damage (approximately 17% and 90%, respectively) rather than by adding it after injury. Furthermore, since NMDA+H_2O_2 could participate in the development of glaucoma through the inhibition of ERK activity ($p < 0.05$ vs. control; Figure 13F), the observation that this was prevented by pre-treatment with VGLCR ($p < 0.05$) or restored by the post-stimulation with the same combination ($p < 0.05$) is an important observation. Finally, it must be emphasized that the pre-treatment with VGLCR can slow down the adverse effects caused by glaucoma inductors better than the post-stimulation ($p < 0.05$ about 70%), and this supports its potential role in counteracting glaucoma.

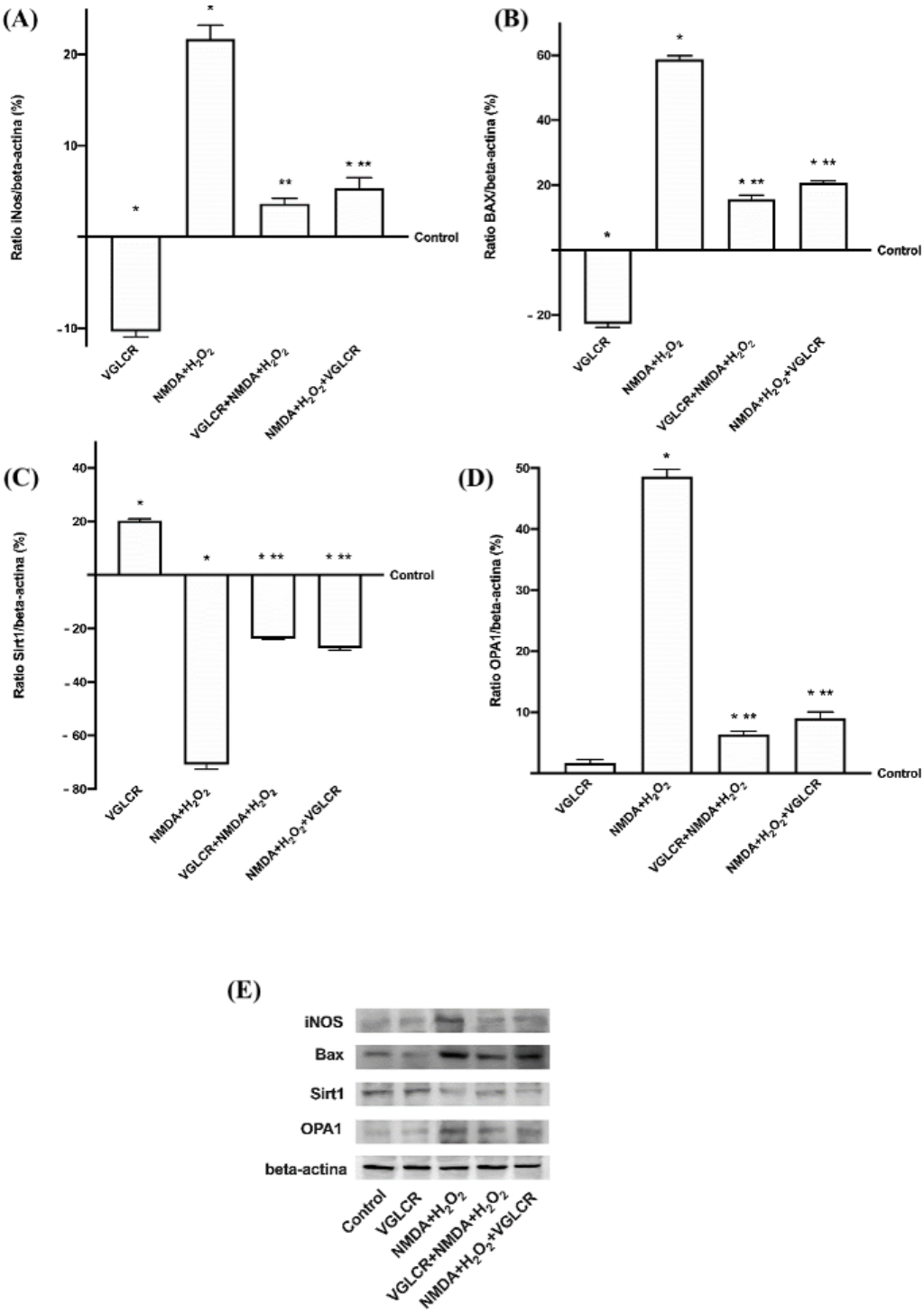

Figure 14. Western blot and densitometric analysis. In (**A**) iNOS, in (**B**) Bax, in (**C**) SIRT1, in (**D**) OPA1, and in (E) Western blot of iNOS, Bax, SIRT1 and OPA1. Retinal tissues were treated with NMDA and H_2O_2 added together, before and after VGLCR stimulations. Images shown are an example of each protein obtained from four independent experiments. Results of 4 independent experiments performed in duplicates are expressed as means ± SD (%) vs control, normalized and verified on β-actin detection. * $p < 0.05$ vs control, ** $p < 0.05$ vs NMDA+H_2O_2; VGLCR+NMDA+H_2O_2 $p < 0.05$ vs NMDA+H_2O_2+VGLCR. Abbreviations are the same as reported in Figure 8.

Ocular damage analysis

To determine the involvement of SIRT1, a marker of ocular aging, and OPA1, a marker of ocular atrophy, Western blot on tissue lysates obtained from eyecup preparations were performed. As shown in Figure 14 C, E, NMDA+H_2O_2 induced a significant decrease in SIRT1 phosphorylation compared to the control ($p < 0.05$), and the pre-treatment with VGLCR was able to reduce this negative effect better than the post-stimulation ($p < 0.05$, about 17%). These data define a novel role for SIRT1 as an important regulator of inflammation or oxidative response under a glaucoma condition. Finally, the glaucoma inductors were able to degrade retinal tissue ($p < 0.05$), but VGLCR pre-treatment was able to counteract this process ($p < 0.05$) by overexpressing OPA1 better than the poststimulation ($p < 0.05$, about 50%), confirming its protective role (Figure 14 D, E). All these findings support the hypothesis that VGLCR can protect the optic nerve, and, consequently, retinal cells by preventing ocular damage. Furthermore, VGLCR was also able to induce a physiological mechanism that allows the slowing of degeneration, as demonstrated by molecular mechanisms.

All data reported are published in: *"Molinari C, Ruga S, Farghali M, Galla R, Fernandez-Godino R, Clemente N, Uberti F. Effects of a New Combination of Natural Extracts on Glaucoma-Related Retinal Degeneration. Foods. 2021 Aug 15;10(8):1885.*

4.3. Project 3: Brain aging

The project aims to investigate on the one hand the role of BDNF as a biomarker of aging and, on the other hand, its activity as a key molecule in maintaining neuronal well-being, also thanks to the synergistic effect obtained combining BDNF with natural substances, such as ginger or vitamin D3.

The potential intestinal absorption as evaluated *in vitro*

Since BDNF can be used by oral administration in humans, the *in vitro* intestinal absorption was investigated. An *in vitro* intestinal barrier model was carried out to understand the ability of 1 pg/mL BDNF compared to 50 ng/mL BDNF to cross the intestinal barrier and to become available to the body. Analyzing the volume in the basolateral compartment 1 pg/mL BDNF showed a significant increase in absorption capacity compared to saline and comparable to that of 50 ng/ml of BDNF at each stimulation time (Table 1). In particular, the maximum Papp, the permeability constant value, was observed at 1 h of treatment with 1 pg/mL BDNF (about 4.18 ± 0.1), supporting the hypothesis that BDNF can cross the intestinal barrier.

Following a standard conversion to predict the human absorption after oral intake starting from the Papp values obtained from Caco-2 cells, the BDNF bioavailability in human (Table 1) showed an increase caused by 1 pg/mL BDNF compared to saline solution and 50 ng/mL BDNF at each time of treatments and confirmed a higher level at 1 h of stimulation (about 4% compared to 50 ng/mL BDNF). These data confirm that 1 pg/mL BDNF is able to cross the intestinal barrier and to have a good bioavailability compared to 50 ng/mL BDNF.

Table 1. The Papp values obtained on intestinal barrier model and plasmatic human absorption derived from Papp value.

Stimulations	30 min	1 h	3 h	4 h	5 h	6 h
1 pg/mL BDNF SKA	2.91 ± 0.3	4.18 ± 0.1	3.36 ± 0.2	3.27 ± 0.3	2.02 ± 0.2	1.97 ± 0.3
Saline	0.11 ± 0.1	0.29 ± 0.1	0.36 ± 0.1	0.5 ± 0.1	0.57 ± 0.1	0.6 ± 0.1
50 ng/mL BDNF	3 ± 0.3	3.98 ± 0.3	4 ± 0.3	3.2 ± 0.2	1.98 ± 0.2	1.02 ± 0.1
1 pg/mL BDNF SKA	32.75	39.8	36.5	34.85	26.6	26
Saline	<20	<20	<20	<20	<20	<20
50 ng/mL BDNF	33.2	38.15	39.8	33.2	25.8	20

Saline= saline solution. Data are expressed as means ± SD (%) of four independent experiments reproduced in triplicates

Since safety is the main problem affecting human use, additional experiments on ROS production were performed on the intestinal barrier to exclude any adverse effects. Both 50 ng/mL BDNF and 1 pg/mL BDNF were able to maintain ROS at physiological levels ($p > 0.05$ vs. saline solution and control), supporting the safe use of this. Besides, the higher effect of 1 pg/mL BDNF compared to 50 ng/mL was observed after 3 and 4 h of treatment ($p < 0.05$; compared to saline solution, 50 ng/mL BDNF and control) demonstrating the best antioxidant action of BDNF.

These data support the hypothesis that BDNF can cross the intestinal barrier and reach the blood in the first 3–4 h after oral intake.

Permeability of BDNF through blood-brain barrier

Since the most important parameter after oral intake is the ability of BDNF to reach the brain tissue, more experiments were performed using the BBB *in vitro* model.

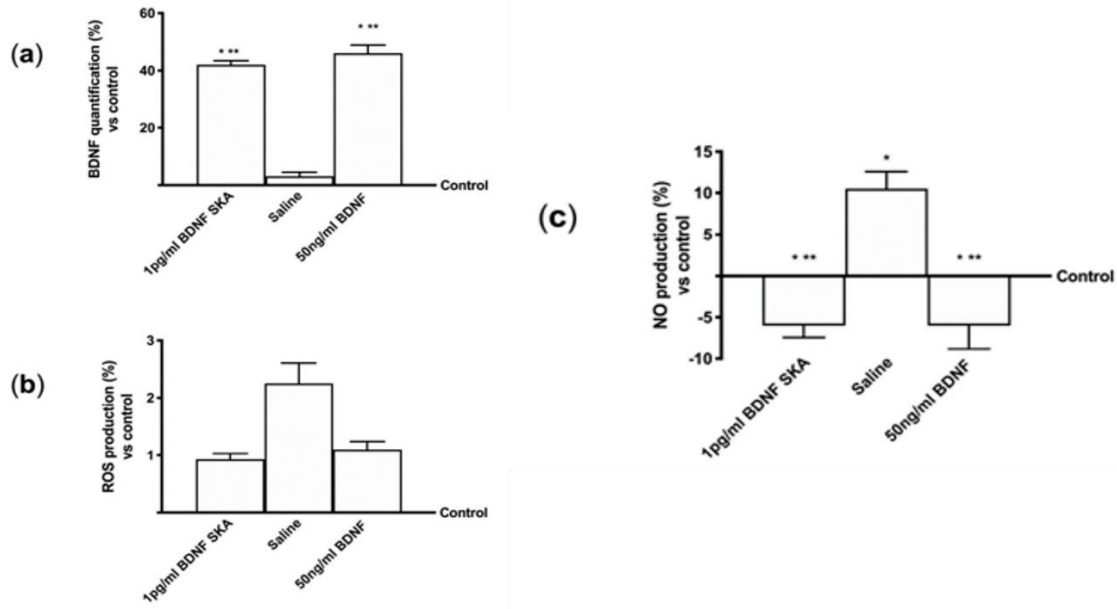

Figure 15. Analysis of the effects at blood-brain barrier (BBB) level. (**a**) Brain-derived neurotrophic factor (BDNF) quantification, (**b**) reactive oxygen species (ROS) production, and (**c**) NO measurements are reported. Data are expressed as means ± SD (%) of four independent experiments performed in triplicates normalized to control (0 line). * $p < 0.05$ vs control; ** $p < 0.05$ vs saline solution.

The analysis on the basolateral volume of 1 pg/mL BDNF and 50 ng/mL BDNF showed no significant difference between 1 pg/mL BDNF and 50 ng/mL BDNF (Figure 15A), but the quantification showed a significant increase ($p < 0.05$) of both 1 pg/mL BDNF and 50 ng/mL

BDNF compared to control and to saline solution (about 41% and about 44%, respectively). These data suggest that only one treatment for six days appears to be more important to obtain a greater effect.

Furthermore, ROS production was analyzed in order to exclude any adverse effect caused by BDNF solutions. As shown in Figure 15B, there was no difference evident between 1 pg/mL BDNF and 50 pg/mL BDNF and the effects of both solutions were not significant ($p > 0.05$) compared to control, indicating a physiological ROS production. These data confirmed the hypothesis of the higher effectiveness of administration protocol.

Since maintaining the balance of oxidative condition is an important parameter to preserve the integrity of brain cells, some additional experiments were carried out to analyze NO production within the BBB (Figure 15C). At basolateral level, the NO production induced by protocol A was significantly reduced compared to control ($p < 0.05$). In particular, there was no significant difference between 1 pg/mL BDNF and 50 ng/mL BDNF treatment, suggesting that BDNF solutions were not cytotoxic.

These results suggest that 1 pg/mL BDNF had a similar effect to 50 ng/mL despite the different concentrations and that only one treatment is able to induce a beneficial effect. Finally, BDNF is confirmed to act without any adverse effect at the neuronal level.

Topic action of BDNF on monolayer culture

BDNF has been demonstrated to be able to act on both cortical neuronal cells and astrocytes, additional experiments were performed in order to investigate cell viability and ROS production in these monolayer cultured cells.

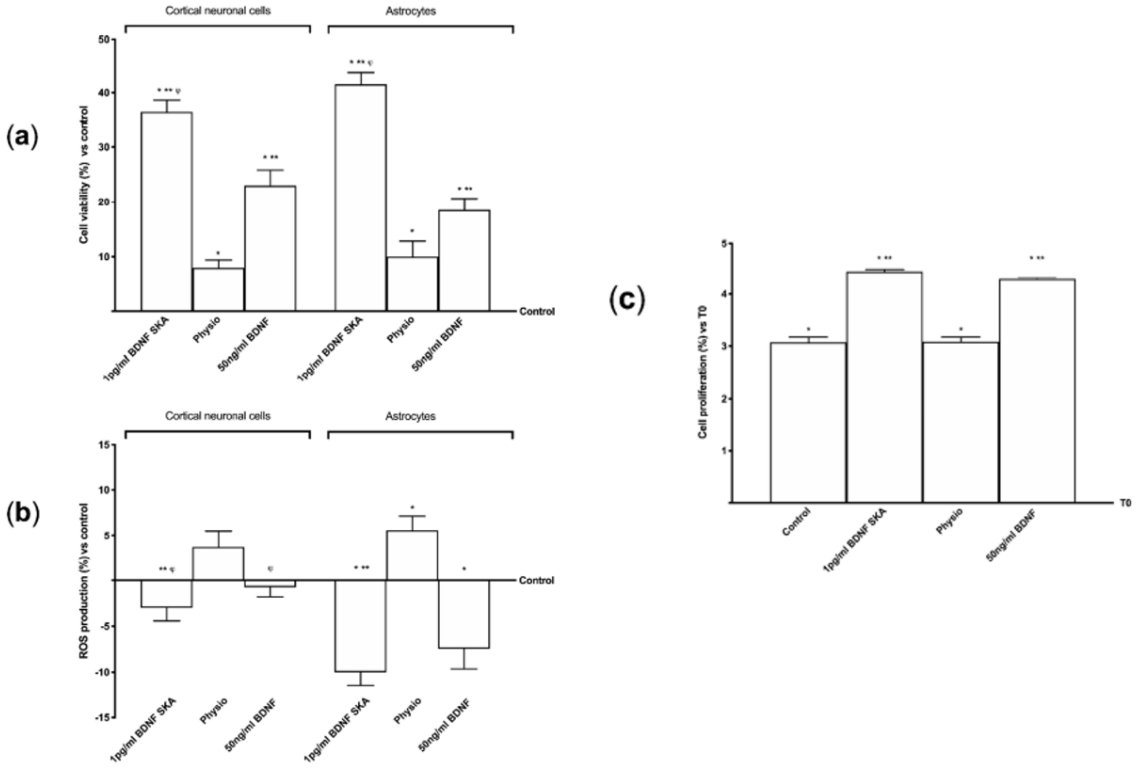

Figure 16. Effects of BDNF solutions on primary cortical neuronal cells and astrocytes. (**a**) Cell viability and (**b**) ROS production measured on both cell types. (**c**) The effects on astrocyte proliferation are shown. Data are expressed as means ± SD (%) of four independent experiments performed in triplicates normalized to control (0 line). * $p < 0.05$ vs control; ** $p < 0.05$ vs saline solution; φ $p < 0.05$ vs the same treatments between primary cortical neuronal cells and astrocytes in the same protocol.

In particular, 1 pg/mL of BDNF induced a significantly greater cell viability (Figure 16A) both in primary cortical neuronal cells and in astrocytes compared to control ($p < 0.05$) and 50 ng/mL of BDNF (in neuronal cells approximately 124% and 75%, respectively, in the astrocytes approximately 58.6% and 171%, respectively). These findings suggest that though BDNF was used at lesser concentrations it was able to determine a greater effect on cell viability compared to the higher concentration, furthermore these results support the hypothesis of the effectiveness of the single administration compared to multiple administrations.

Since an important contributing factor to brain aging is the exaggerated ROS production, additional experiments on ROS production were performed on both cell types following both protocols of treatments (Figure 16B). Results obtained from these experiments show that 1 pg/mL BDNF was able to maintain ROS production within physiological range in both cortical neuronal cells and astrocytes ($p > 0.05$ vs. control). These data suggest that BDNF is able to

maintain the redox balance even in monolayer cultured cells. Although 50 ng/mL BDNF also shows similar properties, the effect was significantly lower compared to 1 pg/mL BDNF. Based on previous observation of viability and ROS production in astrocytes, the involvement of BDNF solutions in cell proliferation was also investigated by crystal violet staining. As reported in Figure 2C, 1 pg/mL BDNF treatment was able to increase the proliferation of astrocytes ($p < 0.05$) compared to control (about 43.9% and 13.4%, respectively) and to saline solution (about 43.5% and 29.6%, respectively). In addition, the importance of the concentration used was confirmed as well. Indeed, 1 pg/mL BDNF is more effective than 50 ng/mL BDNF (about 3.1%).

Intracellular pathways activated by BDNF on monolayer culture

Since all results reported above show a better influence in astrocytes compared to the same protocol on neurons, the intracellular pathways involved were investigated only on astrocytes. In this phase of the study, the intracellular pathways involved in the previously observed effects were studied. The effects induced by 1 pg/mL BDNF on ApoE expression, SIRT1 phosphorylation, ERK/MAPK pathway, and the levels of activation of the BDNF receptor, TrkB, were studied.

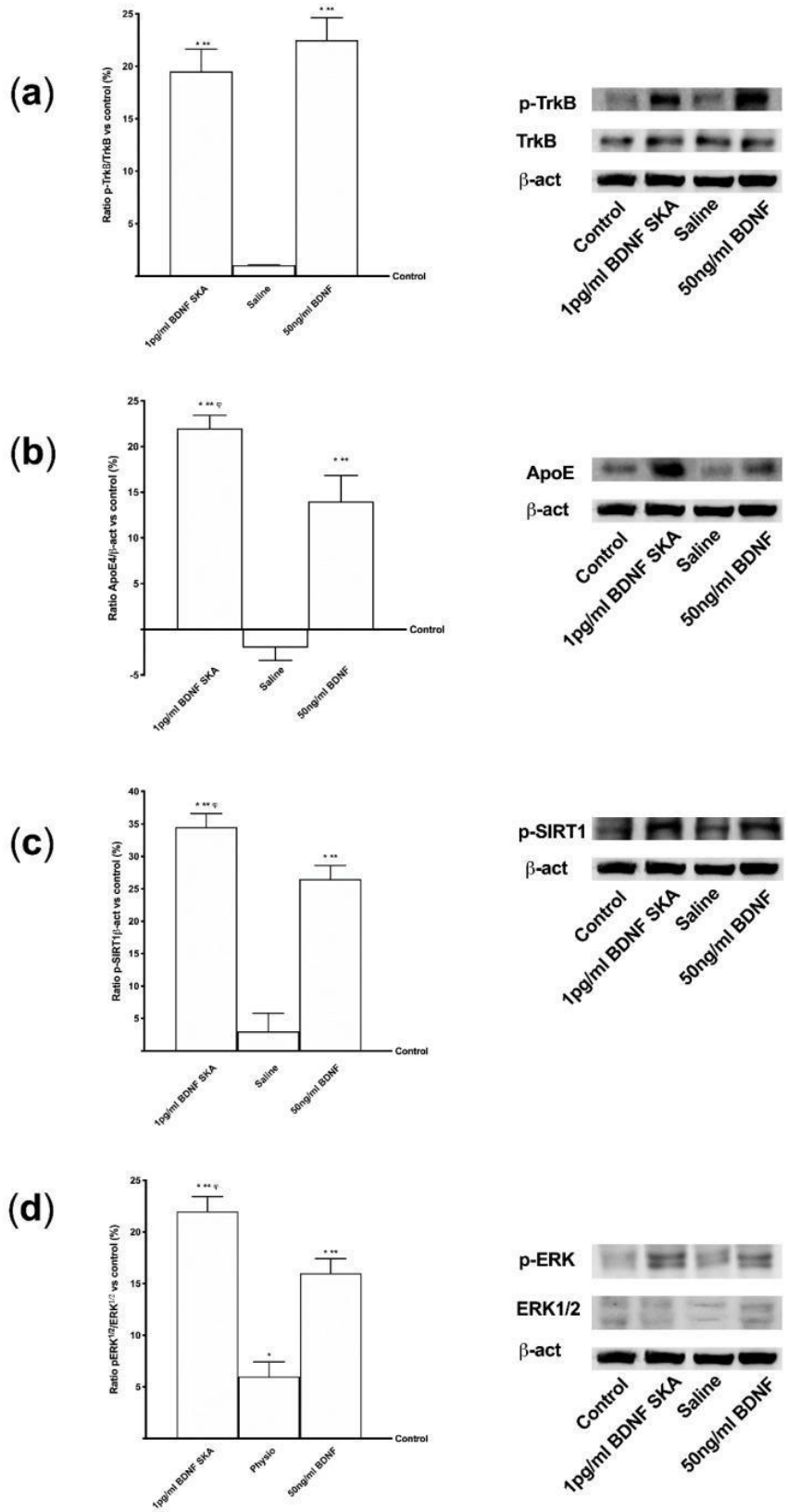

Figure 17. Analysis of intracellular pathways activated by BDNF solutions in astrocytes. In the left side densitometric analysis while in the right side the examples of Western blot are reported. (**a**) TrkB receptor, (b) ApoE (4), (c) SIRT1 and (d) ERK/MAPK expressions are shown. Data are expressed as means ± SD (%) of five independent experiments normalized on specific total protein if possible and verified by β-actin detection. * $p < 0.05$ vs control, ** $p < 0.05$ vs saline solution ; φ $p < 0.05$ vs 50 ng/ml BDNF.

As reported in Figure 17A, BDNF solutions seemed to act by the TrkB receptor. No significant differences were observed between 50 ng/mL BDNF and 1pg/mL BDNF, indicating the ability of 1 pg/mL BDNF that led the TrkB receptor to exert its effects despite the low dose used. As far as the ApoE expression is concerned, it was significantly increased by 1 pg/mL BDNF ($p < 0.05$) compared to 50 ng/mL BDNF (about 80%) (Figure 17B). As illustrated in Figure 17C, phosphorylation of SIRT1 induced by 1 pg/mL BDNF ($p < 0.05$) was increased compared to 50ng/mL BDNF (about 30%), supporting the efficacy of the dosage to increase the presence of this molecule. Finally, as shown in Figure 17D, BDNF has been observed to increase the ERK1/2 expression and the greater effect was obtained with 1 pg/mL BDNF ($p < 0.05$) compared to 50 ng/mL BDNF (about 37%).

Additional experiments were performed to confirm the involvement of the TrkB receptor in previously observed effects, using a pre-treatment with the selective TrkB antagonist ANA-12 (1 µg/mL) on astrocytes. As reported in Figure 18, in the presence of both BDNF solutions, TrkB expression was abolished by the pre-treatment with 1 µg/mL ANA-12, confirming that BDNF solutions acted through the TrkB receptor to explain their effects on astrocytes. These data confirm the importance of the dosage and the protocol of treatment to obtain a beneficial effect on astrocytes under physiological conditions.

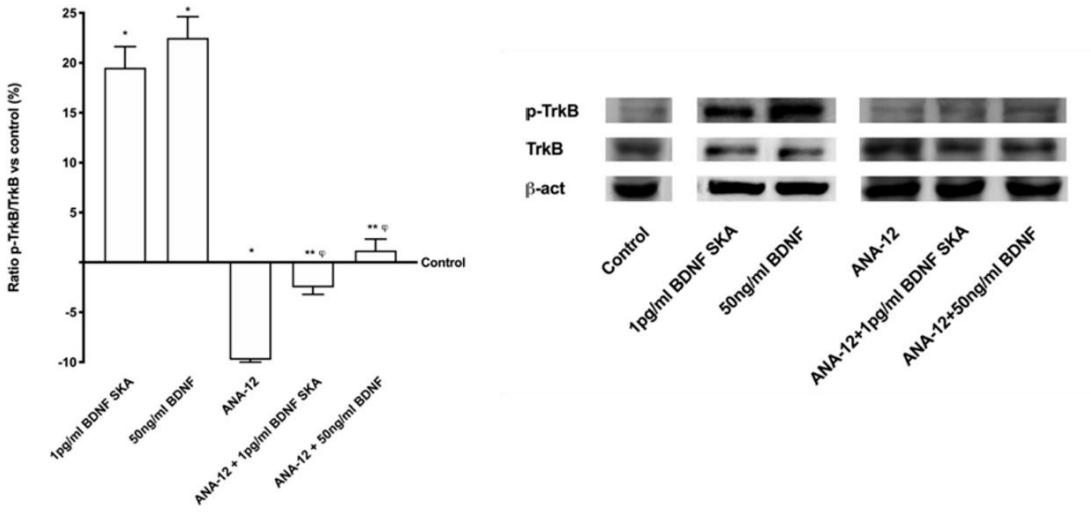

Figure 18. Analysis of TrkB receptor under blocking conditions on astrocytes. In the left panel densitometric analysis and in the right panel an example of Western blot is reported. Data are expressed as means ± SD (%) of five independent experiments normalized on specific total protein and verified by β-actin detection. ANA-12= 1µg/ml. * $p < 0.05$ vs control; ** $p < 0.05$ vs ANA-12; φ $p < 0.05$ vs the same treatment without ANA-12.

Effects of BDNF solutions under oxidative conditions

Cell viability and ROS production were evaluated in cortical neuronal cells and astrocytes in order to understand the potential aging-prevention mechanism of 1 pg/mL BDNF and 50 ng/mL BDNF 50 ng/mL under oxidative conditions. Exposure to 200 µM H_2O_2, in both cell types significantly reduced ($p < 0.05$) cell viability compared to control (Figure 19A), indicating cell loss caused by oxidative injury.

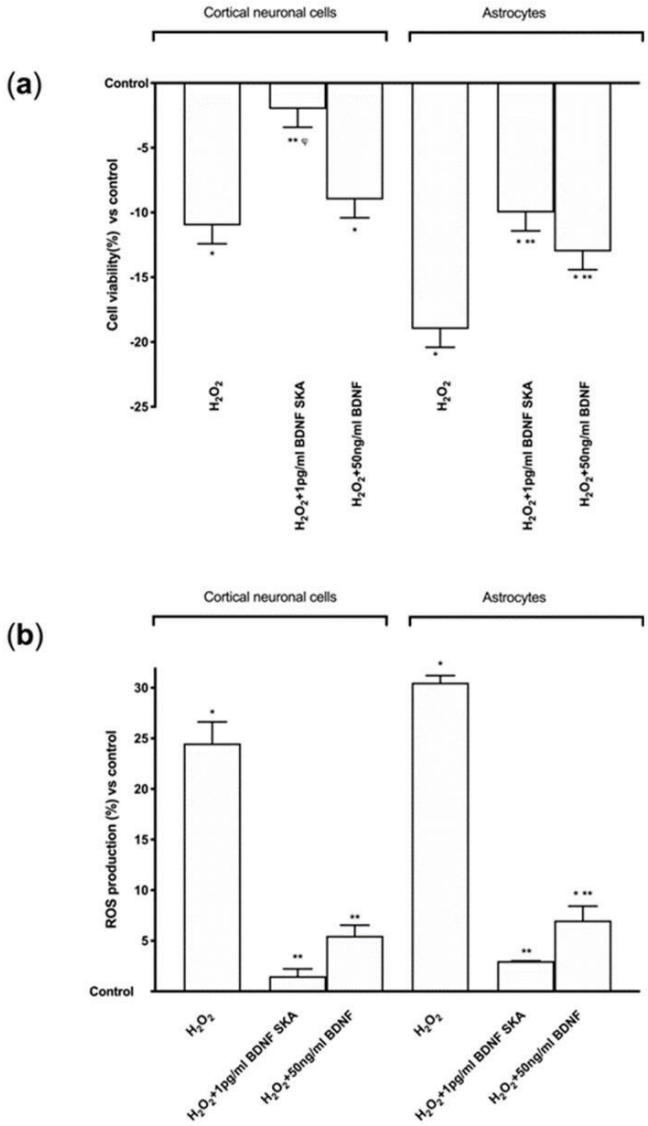

Figure 19. Cell viability and ROS production in both protocols in primary cortical neuronal cells and astrocytes under oxidative condition. (**a**) Cell viability and (**b**) ROS production measured in primary cortical neuronal cells (on the left) and astrocytes (on the right) after treatments. H_2O_2=200µM added 30 min before stimulations. Data are expressed as means ± SD (%) of five independent experiments performed in triplicates normalized to control(0 line). * $p < 0.05$ vs control; ** $p < 0.05$ vs H_2O_2; φ $p < 0.05$ vs H_2O_2+ 50 ng/ml BDNF in the same cells.

Conversely, following post-treatment with 1 pg/mL BDNF and 50 ng/mL BDNF, cell viability increased in a different manner between cell types. Indeed, only in astrocytes did both BDNF solutions significantly increase cell viability ($p < 0.05$), but the main effect was observed with 1 pg/mL BDNF in both cell types ($p < 0.05$ vs. H_2O_2 alone), confirming the importance of doses and posology also, under pathological conditions.

Additional experiments on ROS production were performed. Exposure of cortical neuronal cells and astrocytes to 200 µM H_2O_2 significantly increased the intracellular ROS production compared to control ($p < 0.05$), as illustrated in Figure 19B, confirming the presence of oxidative damage.

Post-treatment with 1 pg/mL BDNF and 50 ng/mL BDNF in both cell types caused a significant reduction of ROS production ($p < 0.05$) compared to H_2O_2 alone, supporting the hypothesis of the importance of BDNF during degeneration to prevent cell loss. These data suggest that 1 pg/mL BDNF is able to counteract the damage induced by oxidative stress with greater effectiveness compared to 50 ng/mL BDNF.

Since the data obtained from the two cell types were comparable, the analysis of intracellular pathways under oxidative conditions were conducted only on astrocytes. BDNF solutions exerted their biological actions by improving TrkB receptor expression even in the presence of H_2O_2, as shown in Figure 20A, ($p < 0.05$ versus control). No significant changes were observed between the two BDNF solutions. Moreover, the expressions of ApoE and of Tau, an important protein that modulates the stability of axonal microtubules, were analyzed. As reported in Figure 20B, the stimulation with H_2O_2 alone caused a significant decrease of ApoE expression compared to control ($p < 0.05$), indicating a loss of neuroplasticity. Conversely, the treatments with 1pg/mL BDNF and 50 ng/mL BDNF repaired the damage and the expression of ApoE was increased. In particular, the main effect was observed by 1 pg/mL BDNF compared to 50 ng/mL BDNF ($p < 0.05$, about tenfold larger). The presence of the damage was confirmed by Tau phosphorylation, as reported in Figure 20C, where the level was even higher than control ($p < 0.05$). After treatment with 1 pg/mL BDNF compared to 50 ng/mL BDNF the phosphorylation was significantly reduced ($p < 0.05$, about 56%), indicating the efficacy of 1 pg/mL BDNF to restore damage. In addition, the analysis of SIRT1 confirms the protection exerted by 1 pg/mL BDNF and 50 ng/mL BDNF against H_2O_2 damage (Figure 20D); 1 pg/mL BDNF and 50 ng/mL BDNF were able to induce a significant increase in SIRT1 phosphorylation compared to H_2O_2 alone ($p < 0.05$) and to control ($p < 0.05$). However, the main effect was shown by 1 pg/mL BDNF compared to 50ng/mL BDNF (about 85%). All these findings support the hypothesis that treatment with BDNF can protect neuronal cells

from the damage induced by the aging process better than high dose BDNF.
Finally, as reportedin Figure 20E, the activation of TrkB induced cells survival by the involvement of ERKs/MAPK; indeed, 1 pg/mL BDNF and 50 ng/mL BDNF added after the injury were able to induce a significant increase on ERK activity compared to H_2O_2 alone ($p < 0.05$) and to control ($p < 0.05$). However, the main effect was observed in the presence of 1 pg/mL BDNF compared to 50 ng/mL BDNF (about 90%).

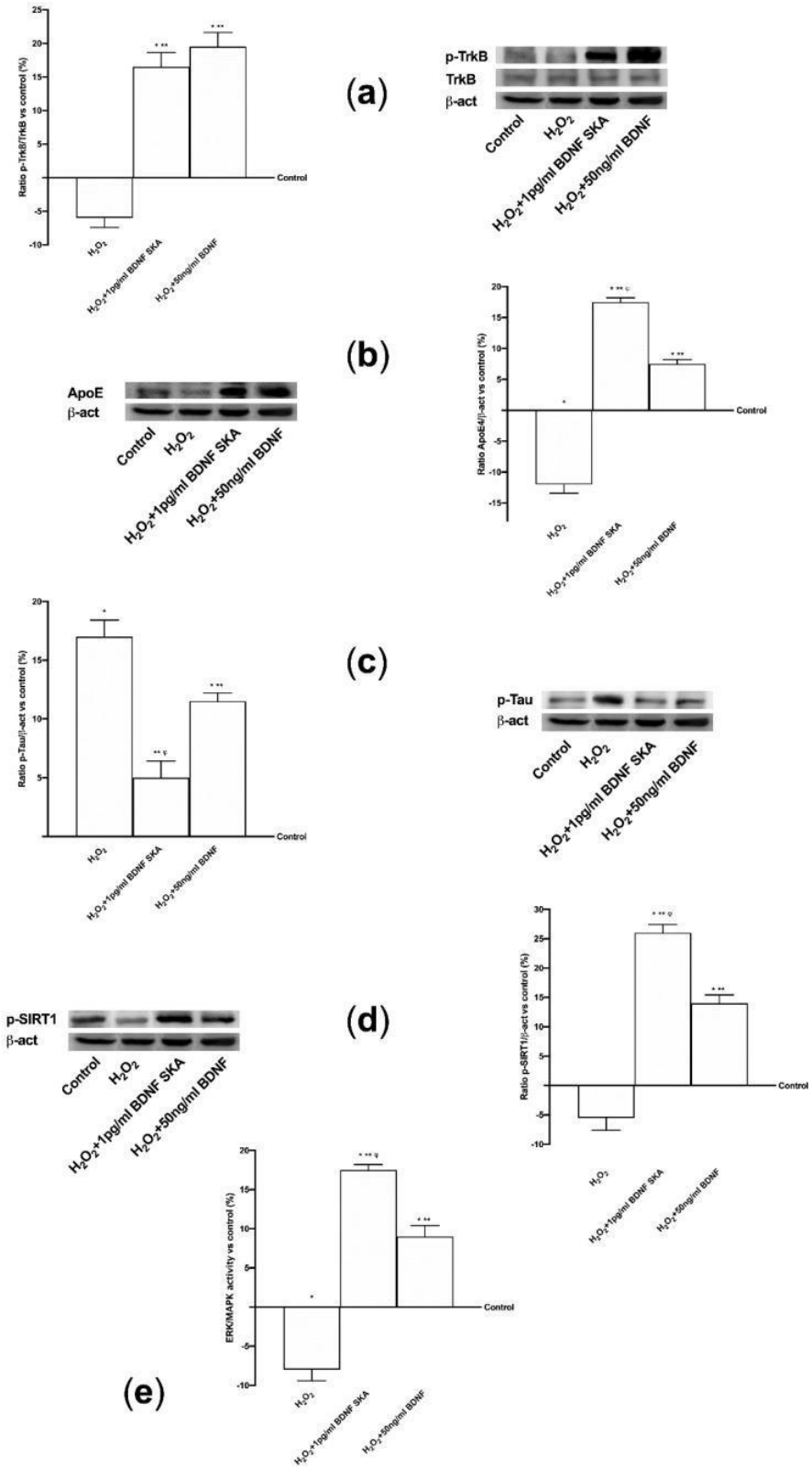

Figure 20. Analysis of intracellular pathways activated by BDNF solutions in astrocytes under oxidative condition. Kinase activity, densitometric analysis and Western blot are reported. (**a**) TrkB receptor, (**b**) ApoE (4), (**c**) p-Tau, and (**d**) SIRT1 expressions and (**e**) ERK/MAPK activity. Data are expressed as means ± SD (%) of five independent experiments and the densitometric analyses are normalized on specific total protein if possible and verified by β-actin detection. * $p < 0.05$ vs control; ** $p < 0.05$ vs H_2O_2; φ $p < 0.05$ vs H_2O_2+50 ng/ml BDNF

Daily duration of the effects of BDNF solutions on neurons and astrocytes

Since BDNF can be used as a dietary supplement in humans, some experiments were carried out to better clarify the optimal dosing schedule. The effects of 1 pg/mL BDNF and 50 ng/mL BDNF during 24 h on both cell types were studied analyzing BDNF concentration, cell viability and mitochondrial potential.

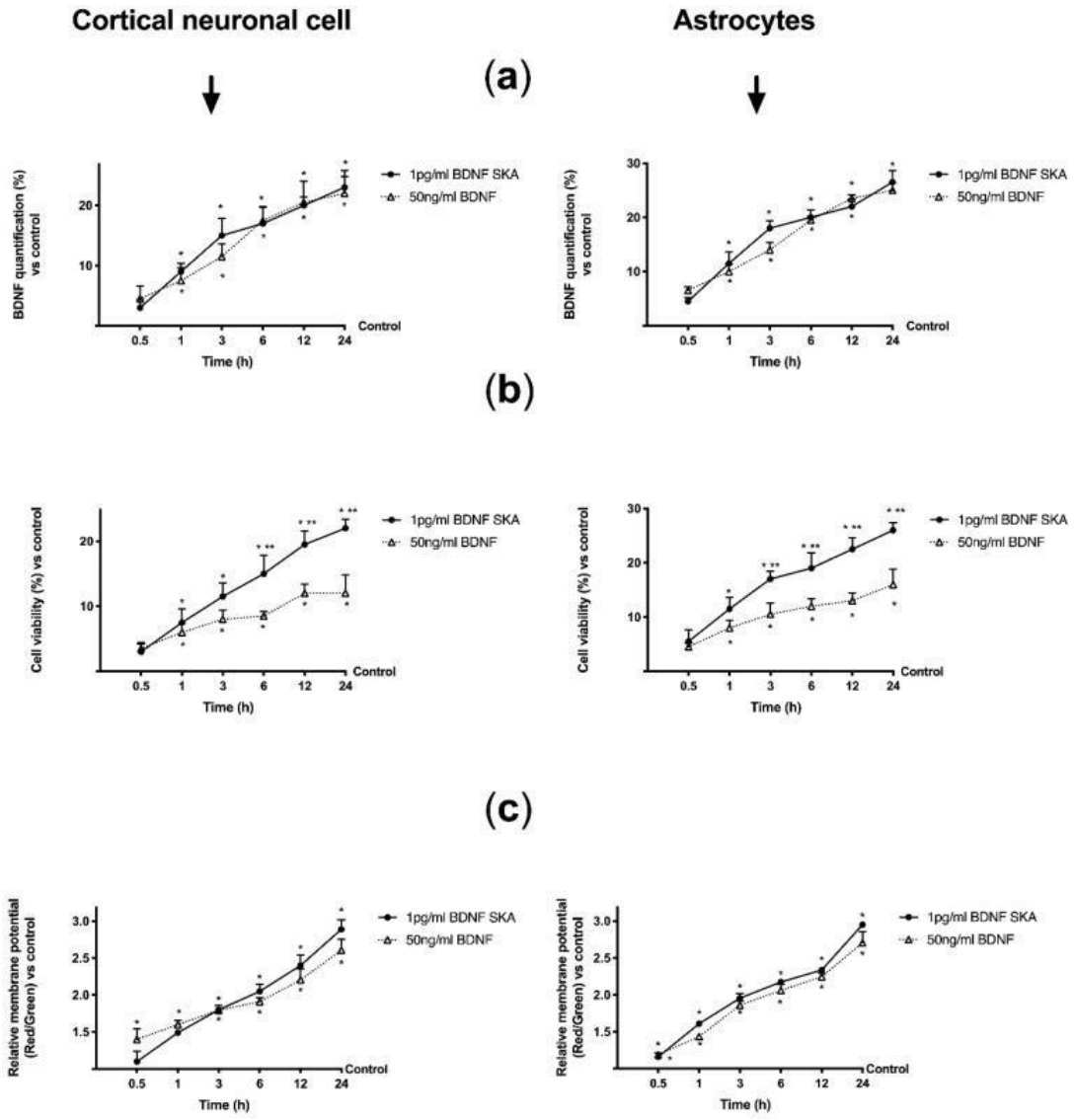

Figure 21. Effects of BDNF solutions within 24 h on primary cortical neuronal cells and astrocytes. In the left column primary cortical neuronal cells and in the right column astrocytes are shown. (a) BDNF quantification and (b) cell viability measured after BDNF treatments. Data are expressed as means ± SD (%) of five independent experiments performed in triplicates normalized to control (0 line as control). (c) the mitochondrial membrane potential is investigated in the same condition. Data are expressed as means ± SD of five independent experiments performed in triplicates normalized to control (1 line as control). * $p < 0.05$ vs control; ** $p < 0.05$ between 1 pg/ml BDNF and 50 ng/ml BDNF

As reported in Figure 21A, treatments with 1 pg/mL BDNF and 50 ng/mL BDNF on both cell types caused a similar time-dependent increase in BDNF concentration. This effect showed significance from 1 h, compared to control ($p < 0.05$), and the maximum effect was observed at 24 h (23% and 22% compared to control, respectively, in neurons; about 26% and 25% compared to control, respectively, in astrocytes). No significant changes between the two BDNF solutions were observed, indicating the comparable effectiveness of low dose to high-concentration BDNF.

However, 1 pg/mL BDNF had better tolerability, demonstrated by better cell viability ($p < 0.05$) compared to 50 ng/mL BDNF. This effect was significant starting from 6 h in neurons and 3 h in astrocytes, with a maximum effect on both types of cells at 24 h (about 80% and about 60% vs. 50 ng/mL, respectively), as shown in Figure 21B.

Since cell viability depends on mitochondrial activity, the analysis of mitochondrial potential variation was performed (Figure 21C). Both BDNF solutions modulated mitochondrial potential in a time-dependent manner with a significant increase from 3 h in neurons and from 30 min in astrocytes compared to control ($p < 0.05$). However, no significant changes were observed between the two BDNF solutions.

These results therefore allow us to state that low dose BDNF administration has more beneficial effects on neurons and astrocytes than high dose BDNF, prolonged over time to cover 24 h.

Analysis of bioavailability of BDNF solutions and their effects in mouse brain

To confirm the ability of BDNF solutions to cross enterohepatic circle and the blood–brain barrier and act in brain tissue, some experiments were performed *in vivo* using wild type C57BL mice. Since in humans the administration would be daily, in some experiments 1.2 pg/mL BDNF and 25 ng/mL BDNF were administered to animals by rissoles and BDNF concentration in both serum and brain tissues were analyzed after 24 h. In addition, to verify the stability of the effects, additional experiments were carried out adding 24 h without stimulations.

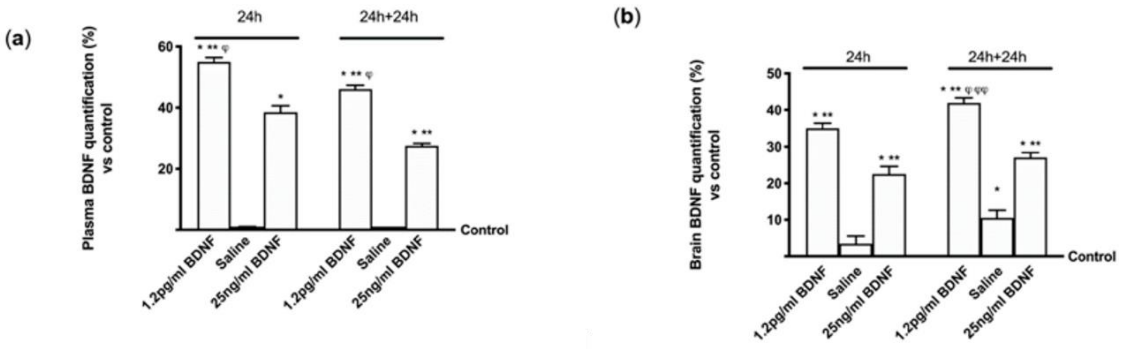

Figure 22. BDNF quantification in mice in serum and brain tissue. (a) serum quantification and (b) BDNF quantification in brain tissue are reported. Each graph contains on the left the results obtained at 24 h (12 animals) and on the right (12 animals) at 24 h plus 24 h (24 h+24 h). Data are expressed as means ± SD (%) normalized to control (0 line as control). * $p < 0.05$ vs control; ** $p < 0.05$ vs saline solution; φ $p < 0.05$ vs the same treatments at the same time of administration; φφ $p < 0.05$ vs the same treatments at 24 h and 24 h plus 24 h.

As reported in Figure 22A, 1.2 pg/mL BDNF had a greater ability to get through the enterohepatic circle compared to 25 ng/mL BDNF (about 43%) and to control ($p < 0.05$) at 24 h. Moreover, 1.2 pg/mL BDNF tended to remain in blood circulation longer (at least 24 h longer), compared to 25 ng/mL BDNF (about 68%). Since BDNF is present in blood, it is important to verify its presence also in brain tissue (Figure 22B). In administration of both 1.2 pg/mL BDNF and 25 ng/mL BDNF, it was able to enter the brain, as illustrated by BDNF quantification analysis ($p < 0.05$ vs. control). In addition, 1.2 pg/mL BDNF was able to remain for a longer time (24 h plus 24 h) in brain tissue compared to 25 ng/mL BDNF (about 55%, $p < 0.05$) and 1.2 pg/mL BDNF at 24 h (about 20%, $p < 0.05$). These findings demonstrate the importance of doses and posology of the administration of BDNF to induce a better influence on brain tissue.

To verify whether the mechanism activated by BDNF solutions is the same as the one observed in cells during *in vitro* experiments, the effects of 1.2 pg/mL BDNF and 25 ng/mL BDNF on some main markers were investigated by Western blot. Since BDNF is necessary for survival of neurons in the brain, after encoding by this gene its expression was investigated, as reported in Figure 23A. 1.2 pg/mL BDNF and 25 ng/mL BDNF both at 24 h and 24 h plus 24 h were able to induce the expression of BDNF compared to control ($p < 0.05$), indicating a better influence of stimulations.

Moreover, 1.2 pg/mL BDNF at 24 h and 24 h plus 24 h caused a significant increase compared to and 25 ng/mL BDNF (about 50% and about 62%, respectively), indicating the induction of endogenous production of BDNF by physiological mechanism, as shown by the significant increase induced by 1.2 pg/mL BDNF at 24 h plus 24 h with respect to at 24 h ($p < 0.05$, about

24%). These effects were mediated by the TrkB receptor, which was expressed in a similar manner in both times of treatment between 1.2 pg/mL BDNF and 25 ng/mL BDNF ($p < 0.05$ vs. control, Figure 23B).

Since β-Amyloid precursor protein (APP) plays a central role, the beneficial effects exerted by both BDNF solutions were also assessed by the quantification of APP, as shown in Figure 23C. 1.2 pg/mL BDNF and 25 ng/mL BDNF increased APP compared to control ($p < 0.05$) at 24 h and 1.2 pg/mL BDNF seemed to have a greater effect compared to 25 ng/mL BDNF (about 2.5 times). In addition, the APP activity at 24 h plus 24 h demonstrated the physiological action of 1.2 pg/mL BDNF compared to 25 ng/mL BDNF ($p < 0.05$), indicating a better regulation exerted by 1.2 pg/mL BDNF on central nervous tissue.

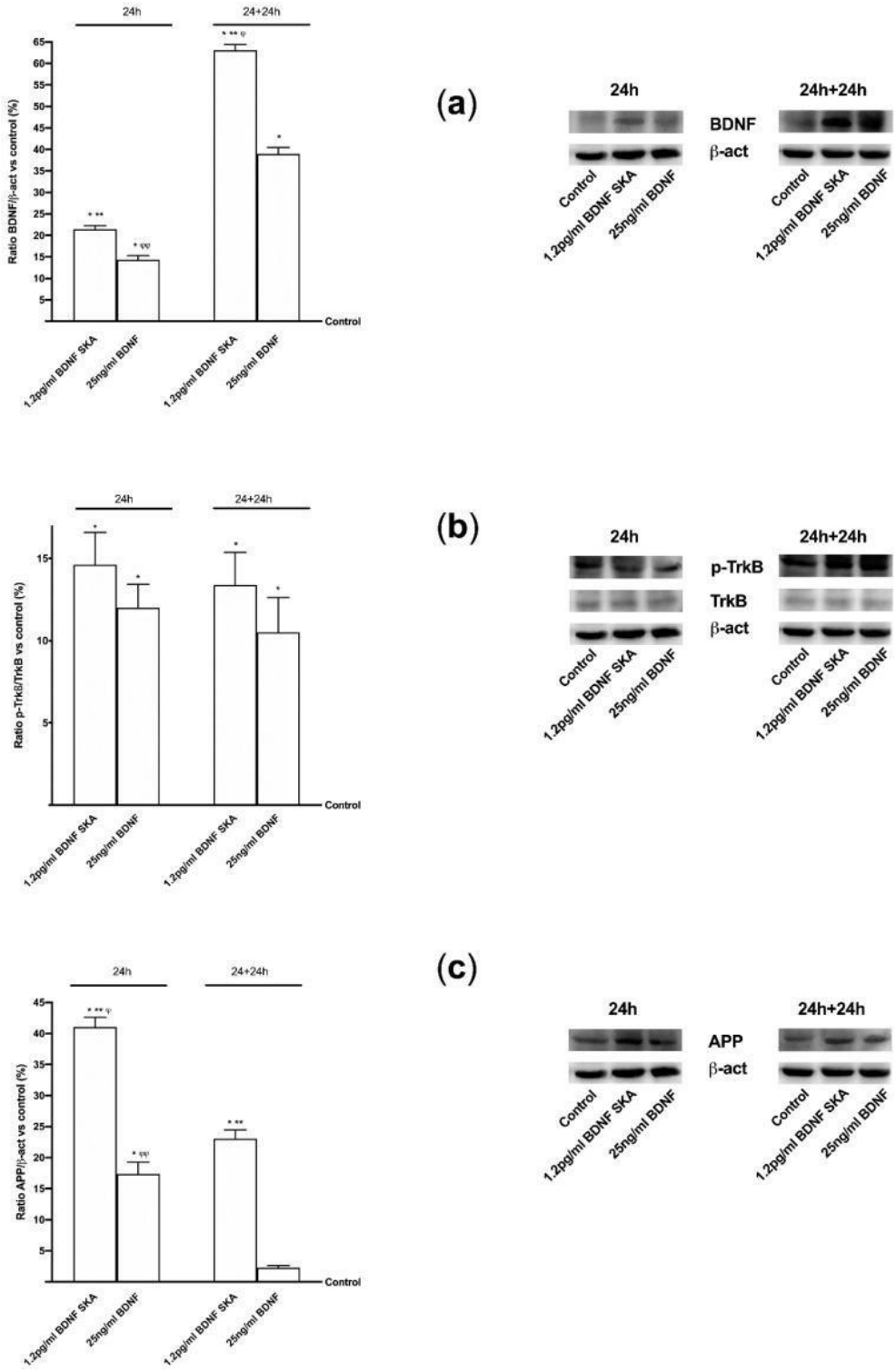

Figure 23. Western blot and densitometric analysis of BDNF protein (a), TrkB receptor (b), and APP protein (c) expressions in brain tissue. In the left column densitometric analysis and in the right column the examples of Western blot are reported. Each group contains on the left the results obtained at 24 h (12 animals) and on the right (12 animals) at 24 h plus 24 h (24 h+24 h). Data are expressed as means ± SD (%) of independent experiments normalized on specific total protein if possible and verified by β-actin detection. * $p < 0.05$ vs control; ** $p < 0.05$ vs 25 ng/ml BDNF at the same time of administration; φ $p < 0.05$ vs 1.2 pg/ml BDNF between 24 h and 24 h plus24 h; φφ $p < 0.05$ vs 25 ng/ml BDNF between 24 h and 24 h plus 24 h.

Moreover, since *in vivo* and *in vitro* studies suggested that ApoE may drive neurodegeneration through an Aβ-dependent mechanism, ApoE expression was assessed as well.

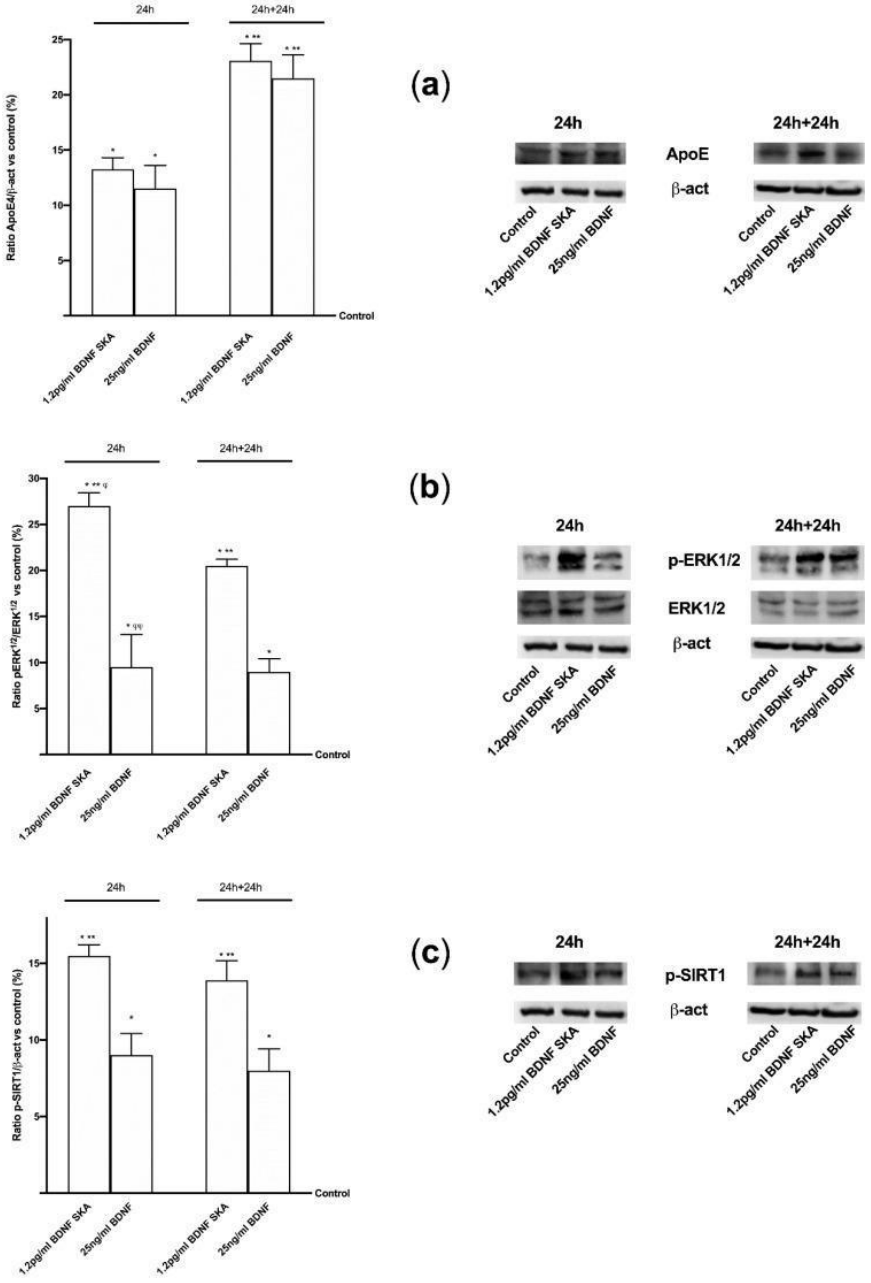

Figure 24. Western blot and densitometric analysis of ApoE (a), ERK/MAPK (b), SIRT1 (c) expressions in brain tissue. In the left column densitometric analysis and in the right the examples of Western blot are reported. Each graph contains on the left the results obtained at 24 h (12 animals) and on the right (12 animals) at 24 h plus 24 h (24 h+24 h). Data are expressed as means ± SD (%) of independent experiments normalized on specific total proteinif possible and verified by β-actin detection. * $p < 0.05$ vs control; ** $p < 0.05$ vs 25 ng/ml BDNF at the same time of administration; φ $p < 0.05$ vs 1.2 pg/ml BDNF between 24 h and 24 h plus 24 h; φφ $p < 0.05$ vs 25 ng/ml BDNF between 24 h and 24 h plus 24 h.

As reported in Figure 24A, a significant increase of ApoE expression compared to control ($p < 0.05$) was observed in the presence of both 1.2 pg/mL BDNF and 25 ng/mL BDNF in both time points, indicating a positive effect of BDNF on central nervous tissue. Moreover, the activation of TrkB by 1.2 pg/mL BDNF and 25 ng/mL BDNF was able to induce a significant increase in ERKs expression compared to control ($p < 0.05$) in both time points. Therefore, a potential role of BDNF in tissue recovery through the involvement of ERKs/MAPK (Figure 24B) can be hypothesized. These findings support what was observed in astrocytes. However, the main effect was observed at 24 h in the presence of 1.2 pg/mL BDNF compared to 25 ng/mL BDNF ($p < 0.05$, about three-fold) and to 1.2 pg/mL BDNF at 24 h plus 24 h ($p < 0.05$, about 30%). The last test of this series of experiments concerned the study of the expression of SIRT1. The analysis of SIRT1 confirms the beneficial effects exerted by 1.2 pg/mL BDNF and 25 ng/mL BDNF (Figure 24C). In both 24 h and 24 plus 24 h, 1.2 pg/mL BDNF and 25 ng/mL BDNF were able to induce a significant increase in SIRT1 phosphorylation compared to control ($p < 0.05$). However, the main effect was shown by 1.2 pg/mL BDNF on both time points compared to 25 ng/mL BDNF (about 70% and 73%, respectively, $p < 0.05$). All these findings support the hypothesis that treatment with BDNF can induce physiological mechanisms potentially able to slow down degeneration and protect the brain during time.

Effects of BDNF solutions in mouse brain during time

To verify whether the efficacy of BDNF was maintained for a long time, the mice were treated following the 6-day protocol previously used in cell experiments.

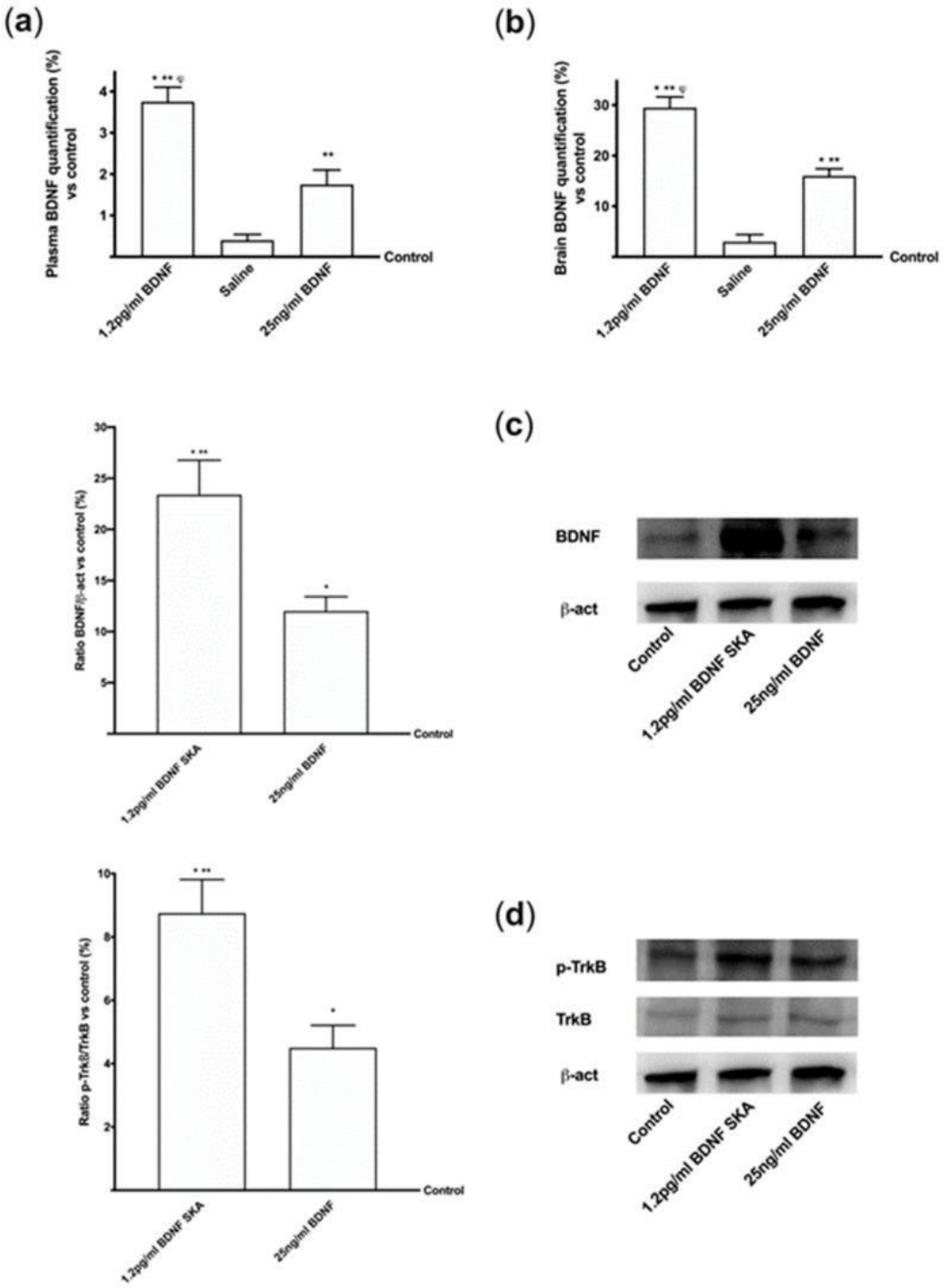

Figure 25. BDNF quantification and intracellular pathways in the brain measured in the 6-days protocol. (a) serum and (b) brain tissue BDNF quantification in mice. Data are expressed as means ± SD (%) of n07 independent experiments normalized to control value (o line). * $p < 0.05$ vs control ; ** $p < 0.05$ vs saline solution ; φ $p < 0.05$ vs 25 ng/ml BDNF. (c) BDNF and (d) TrkB receptor expressions in mice brain reported as densitometric analysis(on the left) and examples of Western blot (on the right). Data are expressed as means ± SD (%) of n=7 independent experiments normalized on specific total protein if possible and verified by β-actin detection. * $p < 0.05$ vs control; ** $p < 0.05$ vs 25 ng/ml BDNF

As reported in Figure 25A, the administration of 1.2 pg/mL BDNF and 25 ng/mL BDNF maintained the serum BDNF levels up to six days compared to control ($p < 0.05$). Besides, 1.2 pg/mL BDNF was able to maintain a high BDNF level compared to 25 ng/mL BDNF (about two-fold higher). Similarly, the administration of 1.2 pg/mL BDNF demonstrated better effectiveness ($p < 0.05$) compared to 25 ng/mL BDNF (about 80%) at brain tissue level (Figure 25B). These data suggest that BDNF tends to remain present for a long time in brain tissue even in the absence of treatment, by triggering its physiological production better than BDNF at a high dose.

To confirm this, some additional experiments were performed to analyze BDNF protein (Figure 25C) and TrkB receptor by Western blot (Figure 25D). Both proteins show a significant increase at six days after both BDNF solutions, but 1.2 pg/mL BDNF exerted a significant increase compared to 25 ng/mL BDNF (about two-fold higher for each one, respectively, $p < 0.05$). These findings support the hypothesis of a fine endogenous regulation exerted by BDNF on brain maintenance and function.

Data regarding BDNF role are published in: “Results obtained were published in the following article: *Molinari C, Morsanuto V, Ruga S, Notte F, Farghali M, Galla R, Uberti F. The Role of BDNF on Aging-Modulation Markers. Brain Sci. 2020 May 9;10(5):285.”*

Starting from these results, it was decided to analyze the possible mechanism of endogenous induction of BDNF production in neuronal cells.

In this context the neuroprotective effect of 6-shogaol (Ginger extract) was analyzed also in combination with Vitamin D3 and curcumin in both OS condition and aging degenerative process. Vitamin D3 has been long known for its important role in calcium homeostasis for bone health, however, its novel role in the brain has been related to the presence of enzymes for both the anabolism and catabolism in the brain which shows its brain localization. Several functions have been highlighted in the brain, such as stimulating neurogenesis due to its proliferative function, activating neurotrophic factors. vitamin D3 has shown promising effects in protecting against oxidative stress by increasing the activity of antioxidants defenses, neuroinflammation, providing neuroprotection during aging and neurodegenerative diseases [418].

Multiple studies have shown the various roles of curcumin as a natural product, such as a free radical scavenger and antioxidant, immunoregulatory agent that can modulate the activation and function of T-cells, efficiently reducing the secretion of pro-inflammatory cytokines. Recent experiments have demonstrated that it protects dopaminergic neurons against mediated neurotoxicity, limits brain inflammation and rescues from cell death [419].

According to the results from studies on the prevention of neurodegenerative diseases using curcumin and vitamin D3, they have shown high synergistic effects through antioxidants and anti-inflammatory mechanisms. The experiments performed and reported above show the effects of curcumin, vitamin D3 and 6-shogaol alone and combined to explore a possible synergic effect to reduce the oxidative stress condition leading to neurodegeneration, analyzing several inflammatory and stressor markers.

Dose-response and time-course study on SH-SY5Y cell line

SH-SY5Y cell line [420] was treated with 6-shogaol (S, 5µM-20µM) in a dose-dependent and time-course study (from 24 h to 72h) to explore the better concentration to use. Since 1nM Vitamin D3 (V) and 20µM Curcumin (C) were previously analyzed [382], these concentrations were maintained on all experiments. Mitochondrial metabolism was analyzed by MTT assay to verify the minimum effective concentration and to exclude any cytotoxic effects. As shown in Fig.26 (panel A), all concentrations tested were able to maintain mitochondrial metabolism compared to the control ($p < 0.05$) without cytotoxicity. In particular, S 5µM showed the best significant effects ($p < 0.05$) compared to the other concentrations tested (about 77.35% compared to S20 and 10.55% compared to S10) and it was maintained in all successive experiments. Since Curcumin and 6-shagol could be used as dietary supplements and its effect could be amplified [306;342], additional experiments were performed testing C 20µM, V 1nM, and S 5µM alone and combined through analyzing cell viability after 24 h of stimulation. As shown in panel B, all these substances tested alone were able to induce a significant increase in mitochondrial metabolism ($p < 0.05$) compared to the control; in addition, the combination of C 20µM, V 1nM, and S 5µM, named CVS, seem to have a synergistic effect. In particular, CVS had effects greater than single agents about 56.7%% compared to C, about 38.2% compared to V and about 39% compared to S ($p < 0.05$). All these data support the hypothesis of the use of the combination without side effects. For this reason, only the CVS combination was tested in all subsequent experiments.

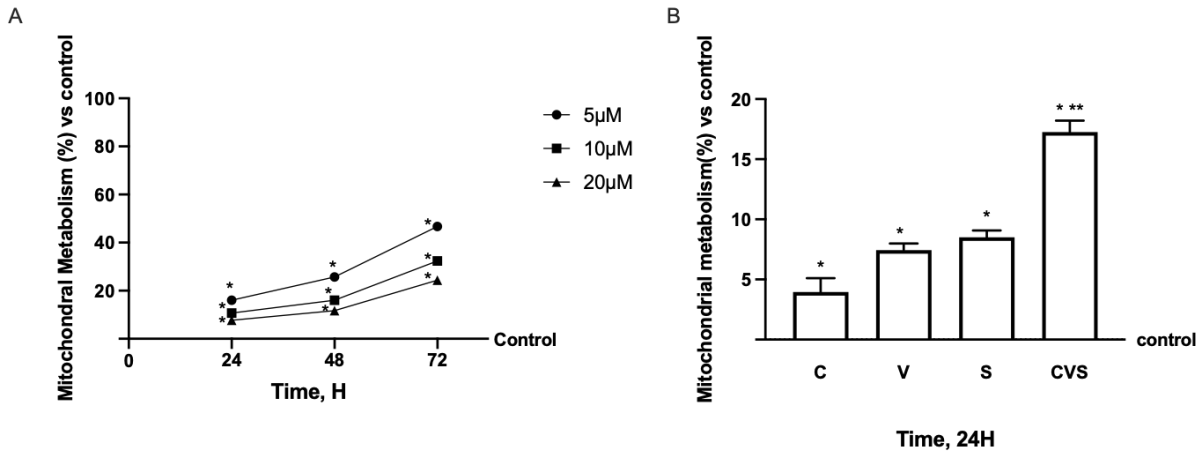

Figure 26. Mitochondrial metabolism in SH-SY5Y cell line measured by MTT assay. In A S=6-shogaol and in B the better concentration of S, Curcumin (C) and Vitamin D3 (V) alone and combined (CVS) were reported at 24 h. Data reported are expressed as means ± SD (%) vs. control (0% line) of five independent experiments, each performed in triplicate. *p < 0.05 vs control; **p < 0.05 vs all single agents

Cell viability under brain aging conditions

Since CVS may be used to slow down brain aging, some experiments on SH-SY5Y cells were performed in order to investigate the potential action to prevent the damage caused by H_2O_2 and Fe^{3+}, agents well known to mimic brain aging conditions. As shown in figure 27, both 100µM H_2O_2 and 75µM Fe^{3+} confirmed their negative effects on SH-SY5Y cells reducing cell viability ($p < 0.05$ compared to control), indicating the presence of damage. Conversely, following the treatment with CVS mitochondrial metabolism was increased: CVS is able to revert cell loss improving mitochondrial metabolism compared to control (about 3.84%) and compared to 100µM H_2O_2 and 75µM Fe^{3+} (about 20.95% and 24.8% respectively) ($p < 0.05$).

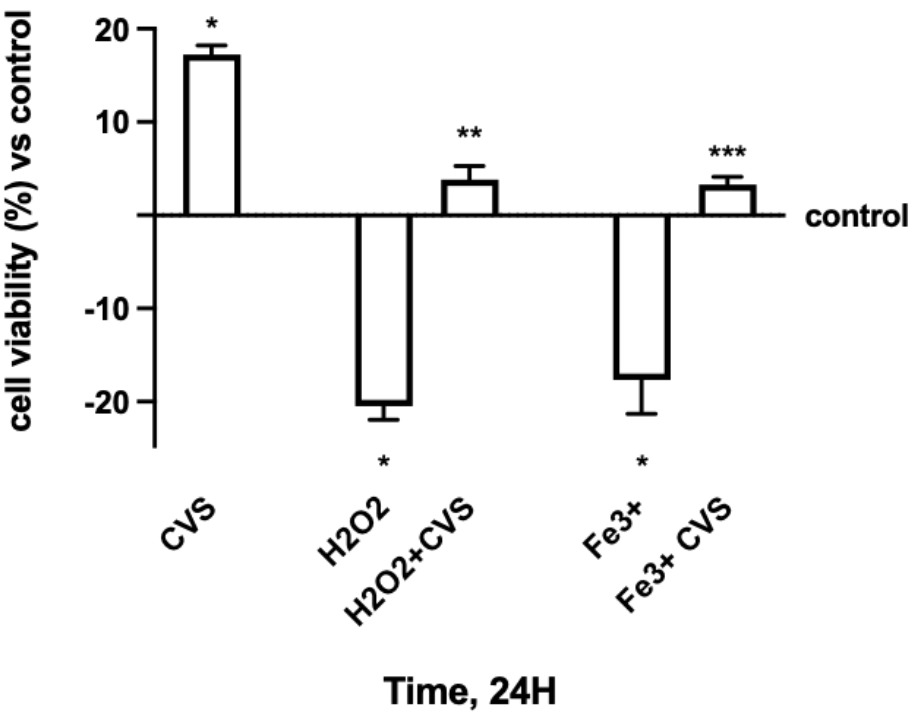

Figure 27. Cell viability of CVS on SH-SY5Y cell line under H_2O_2-induced oxidative stress and iron -induced damage conditions. The abbreviations are the same as reported in Figure 26. Data represented are expressed as means ± SD (%) vs. control (0% line) of five independent experiments, each performed in triplicate. *$p < 0.05$ vs control, **$p < 0.05$ vs H_2O_2, ***$p < 0.05$ vs Fe^{3+}

Since the main theory at the basis of brain aging regards the oxidative condition, additional experiments on ROS production were performed. As shown in Figure 28, 100µM H_2O_2 and 75µM Fe^{3+} confirmed the data obtained with MTT test by improving ROS production and indicating cell loss; on the contrary, CVS is able to maintain the ROS production under the physiological level (about $p > 0.05$), supporting the hypothesis of its beneficial effect. In addition, the treatment with CVS after the damages induced by both 100µM H_2O_2 and 75µM Fe^{3+} can reduce ROS production leading to control values (about 1.5 times compared to 100µM H_2O_2 and about 3.5 times to 75µM Fe^{3+}, ($p < 0.05$ on both conditions tested). All these findings represent the ability of the combination to counteract the iron and hydrogen peroxide damage, preventing brain aging.

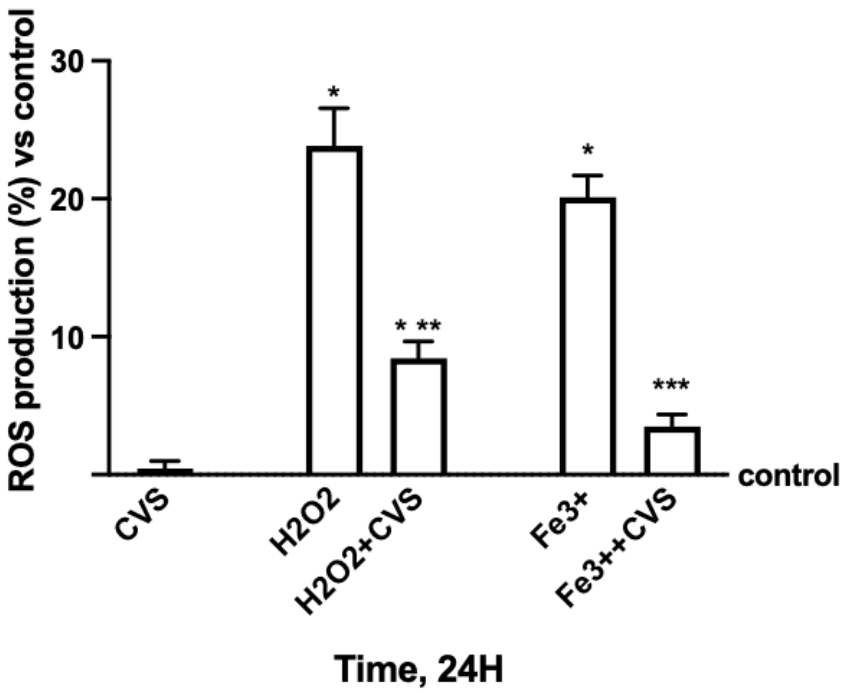

Figure 28. ROS production of CVS stimulation on SH-SY5Y cell line. The abbreviations are the same as reported in Figure 27. Data represented are expressed as means ± SD (%) vs. control (0% line) of five independent experiments, each performed in triplicate. *$p < 0.05$ vs control, **$p < 0.05$ vs H_2O_2, ***$p < 0.05$ vs Fe^{3+}

CVS effectiveness during brain aging process

The improvement of ROS production, caused by oxidative stress and iron accumulation, during aging contribute to cell damage [14]. In this context, in order to confirm CVS property to revert oxidative damage and slow down brain aging, SOD3 and iNOS expressions were also investigated by ELISA test. As reported in figure 29, the SOD3 and iNOS activities were significantly increased in the presence of H_2O_2 or Fe^{3+} (about 18.21% compared to control, $p < 0.05$), supporting the hypothesis of the involvement of oxidative stress in brain aging. As expected, CVS significantly reduced the activity of SOD3 compared to H_2O_2, about 10.52%, and Fe^{3+}, about 10.66%, indicating a beneficial effect in counteracting the aging process. In particular, as shown in Figure 29b, H_2O_2 and Fe^{3+} improved iNOS activity ($p < 0.05$ compared to the control), but CVS treatment was able to reverse this condition supporting its antioxidant potential.

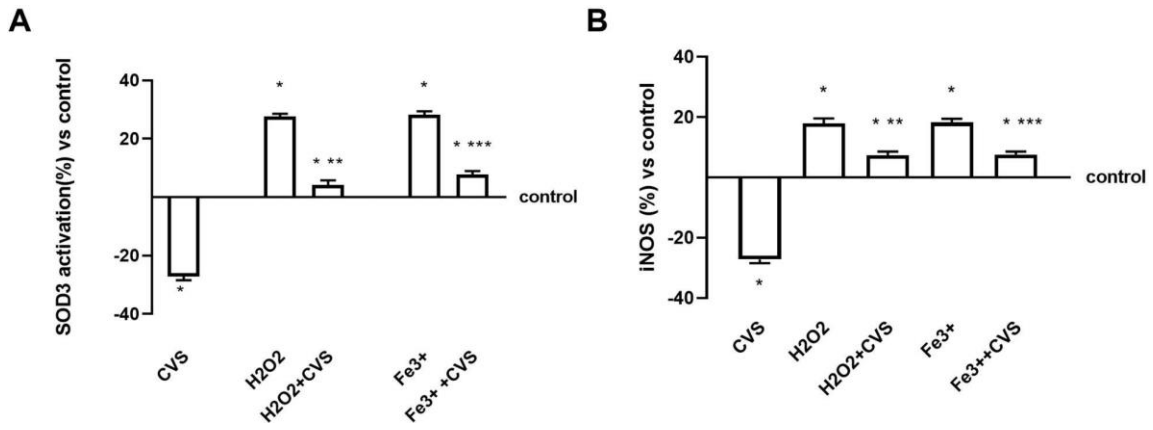

Figure 29. SOD3 and iNOS activity measured by ELISA test in SH-SY5Y cell line. The abbreviations are the same as reported in Figure 27. Data represented are expressed as means ± SD (%) vs. control (0% line) of five independent experiments, each performed in triplicate. *$p < 0.05$ vs control, **$p < 0.05$ vs H_2O_2, ***$p < 0.05$ vs Fe^{3+}

Since oxidative stress is considered as a key factor for brain aging, additional experiments were carried out in order to evaluate the effectiveness of CVS to slow down brain aging process [244] analyzing several intracellular markers; in particular, SIRT1 which is widely recognized to have a role in neuroprotection against neurodegeneration progression, APP which is implicated in neurogenesis, beta-amyloid which contributes to aging-dependent synapse loss and neurodegeneration from the deposition of misfolded amyloid protein, and pTau which is an important marker to aging and neurodegeneration during oxidative stress and age-related disorders. As shown in figure 30 (panel A), 100µM H_2O_2 and 75µM Fe^{3+} induced a significant decrease in sirt-1 expression ($p < 0.05$ compared to the control) as a sign of increased stress and inflammation; the CVS treatment increases this reduced expression ($p < 0.05$, about 5.2% compared to H_2O_2 and about 10% compared to Fe^{3+}) confirming its positive role in reducing brain oxidative damage. Since β-Amyloid protein and APP play a central role in neurodegeneration, further experiments were carried out in presence of 100µM H_2O_2 and 75µM Fe^{3+} and, as shown in figure 30 (panel B and C), hydrogen peroxide and iron treatment improve proteins expressions. Conversely, CVS slightly ameliorated the increase in their expressions (about 49.5% compared to H_2O_2 and about 94% compared to Fe^{3+}, $p < 0.05$). Finally, in order to confirm CVS's beneficial effect, p-Tau expression was evaluated since it is an important marker in neurodegenerative diseases and modulates the stability of axonal microtubules. Data reported in figure 30 (panel D) confirm CVS as a potential treatment to counteract brain oxidative damage since it is able to reduce 100µM H_2O_2 and 75µM Fe^{3+} negative effects (about 14.99% and 17.43% respectively, $p < 0.05$).

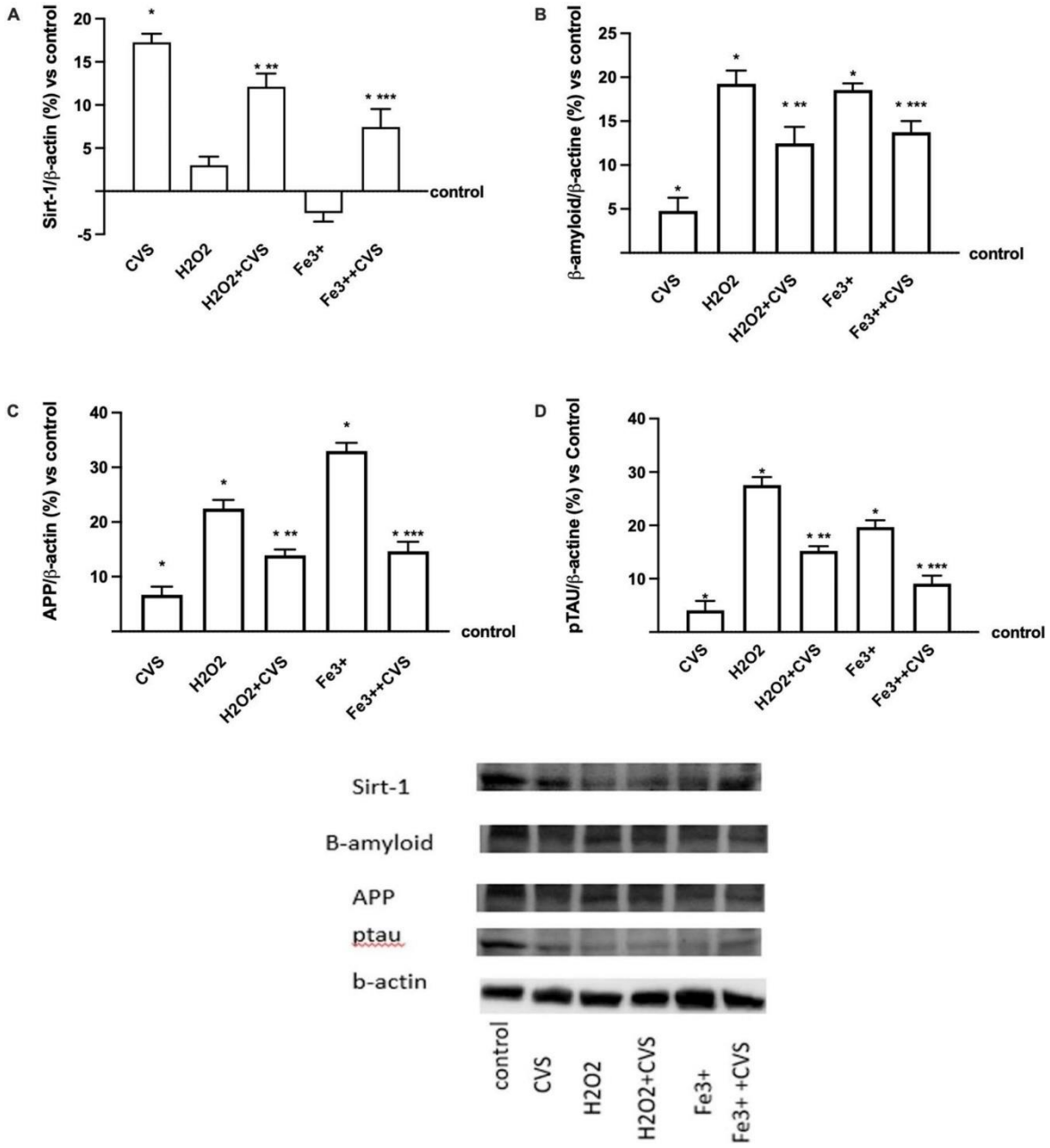

Figure 30. Sirt1 (panel A), β-amyloid (panel B), APP (panel C) and p-tau (panel D) expressions analyzed by Western blot and densitometric analysis on SH-SY5Y cell line. The abbreviations are the same as reported in Figure 27. Data represented are expressed as means ± SD (%) vs. control (0% line) of five independent experiments, each performed in triplicate. *p < 0.05 vs control, **p < 0.05 vs H_2O_2, ***p < 0.05 vs Fe^{3+}

BDNF quantification in astrocytes treated with CVS

In order to evaluate if CVS is able to stimulate BDNF production, additional experiments were carried out on astrocytes to analyze its physiological effects; astrocytes were treated with CVS alone or after pretreated with 100µM H_2O_2 for 30 min or for 24 h with 75µM Fe^{3+}, the astrocyte-conditioned medium was later used to treat SH-SY5Y cells for 24 h [385] in order to mimic the neurodegenerative process [386]. Astrocytes were treated with exogenous BDNF (1 pg/ml) as positive control. BDNF production was quantified using ELISA kit along with analyzing the concentration of BDNF in extracellular and intracellular environments. As shown in Figure 31A, CVS treatment under physiological conditions had induced a slight BDNF production ($p < 0.05$) compared to control and exogenous BDNF (about 43% and about 92% respectively). In the presence of 100µM H_2O_2 or 75µM Fe^{3+}, BDNF production was inhibited as a sign of damage; however, such inhibition was counteracted after 24 h CVS treatment ($p < 0.05$) compared to control and exogenous BDNF (about 31%, 28% and about 13%, 14.1% respectively), confirming our hypothesis of CVS effectiveness in slowing down aging process. Based on this result showing that astrocytes treated with CVS combination had induced BDNF profile, the astrocytes-conditioned medium was further applied on SH-SY5Y cell line to determine the possibility to stimulate endogenous BDNF production, also in presence of 100µM H_2O_2 or 75µM Fe^{3+}. As shown in figure 31 B, SH-SY5Y cells showed an induction in BDNF production after the changed medium at 24 h post-conditioned medium in the extracellular environment more than the intracellular site under the physiological condition. Also in this case, 100µM H_2O_2 or 75µM Fe^{3+} induce damage compared to control ($p < 0.05$) however, the post stimulation with CVS can revert this condition (about 40% compared to H_2O_2 and about 90% compared to Fe^{3+}, $p < 0.05$), confirming its positive role to improve BDNF production and slow down aging process.

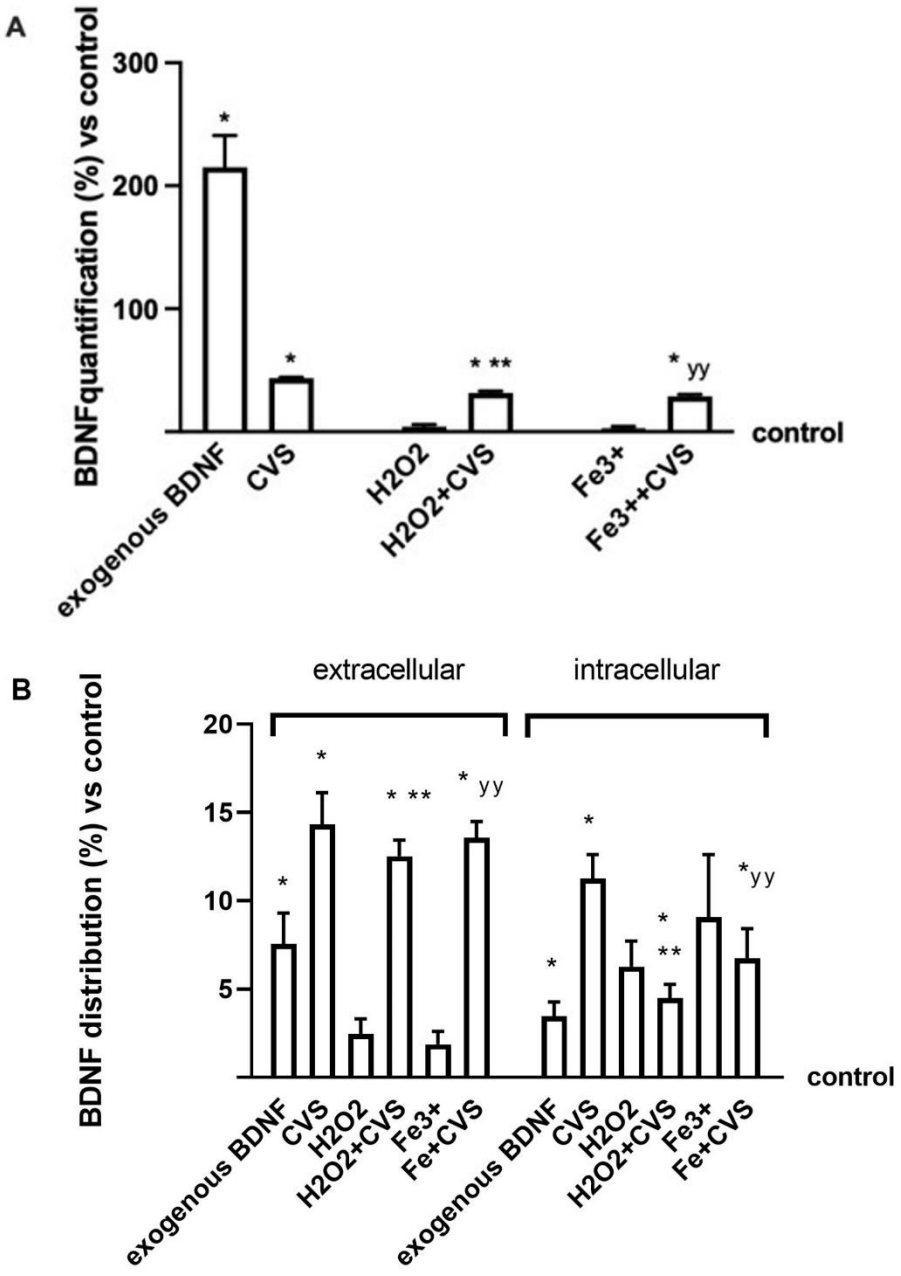

Figure 31. In panel A, BDNF quantification in astrocytes; in panel B cellular distribution of induced BDNF either extracellular or intracellular in SH-SY5Y cell line. The abbreviations are the same as reported in Figure 27. Data represented are expressed as means ± SD (%) vs. control (0% line) of five independent experiments, each performed in triplicate. *p < 0.05 vs control, **p < 0.0.5 vs H_2O_2, yy p < 0.05 vs Fe^{3+}.

Analysis of the main intracellular pathways activated in both astrocytes and human neuroblastoma SH-SY5Y

To confirm the ability of CVS to stimulate BDNF production, further experiments were performed using an astrocyte conditioned medium on SH-SY5Y and analyzing the main intracellular pathways involved in brain aging after changing medium for 24 h. In this context, proBDNF precursor and TrkB receptor were evaluated. As reported in figure 32, 100µM H_2O_2 and 75µM Fe^{3+} demonstrated the reduction of proBDNF precursor expression and consequently TrkB was switched off (about 16% and 19% respectively compared to control, $p < 0.05$). Conversely, CVS post treatment was able to improve pro-BDNF precursor expression in oxidative stress and iron- induced damage (about 27% compared to H_2O_2 and about 15% compared to Fe^{3+}) indicating the beneficial effect of CVS to stimulate BDNF production. Consequently, CVS is also able to activate BDNF receptor during oxidative stress and iron-induced damage (about 28% compared to H_2O_2 and about 14.7% compared to Fe^{3+}, $p < 0.05$) confirming the hypothesis that CVS can maintain the interaction between astrocyte and neuron and stimulate BDNF production.

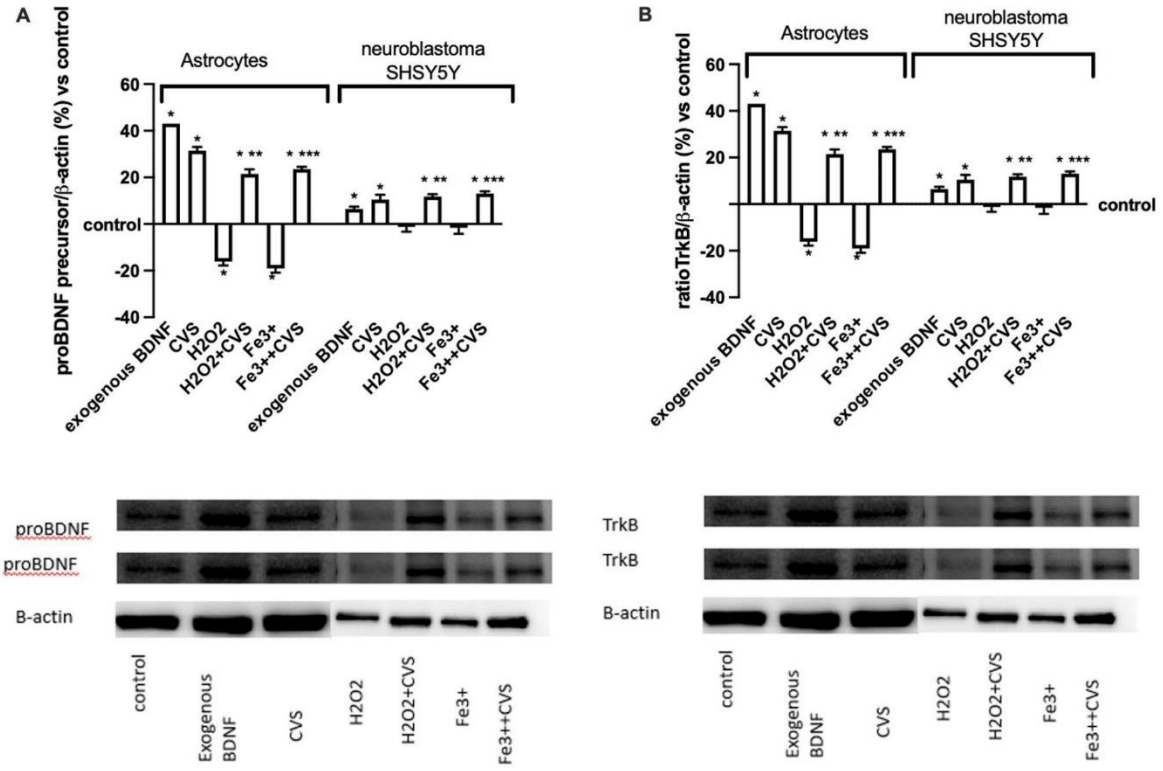

Figure 32. Western blot and densitometric analysis of (A) proBDNF precursor, and (B) TrkB receptor in both astrocytes and SH-SY5Y. The abbreviations are the same as reported in Figure 27. Data represented are expressedas means ± SD (%) vs. control (0% line) of five independent experiments, each performed in triplicate. *$p < 0.05$ vs control, **$p < 0.05$ vs H_2O_2, ***$p < 0.05$ vs Fe^{3+}.

Since CVS can produce BDNF and maintain the connection from astrocyte to neuron, additional experiments were carried out to confirm the potential role of CVS to slow down brain aging process analyzing APP, β-amyloid, SIRT1 and pTau maintained the same experimental condition. As shown in Figure 33A, 100µM H_2O_2 and 75µM Fe^{3+} confirming previously Western blot analysis, in which oxidative stress and iron-induced damage produced a dramatic alteration in brain protein function. In particular, APP and β-amyloid improved their expressions compared to control ($p < 0.05$); conversely, the presence of CVD reduced these increases ($p < 0.05$). In particular, CVS increased APP compared to control and exogenous BDNF (about 7.9% and 5.2% respectively, $p < 0.05$) in both astrocytes and SH-SY5Y indicating better regulation on the central nervous system. Additionally, in Figure 33 B, β-Amyloid protein expression has shown a similar expression profile as APP in both astrocytes and neuroblastoma SH-SY5Y in the different conditions compared to control and exogenous BDNF ($p < 0.05$).

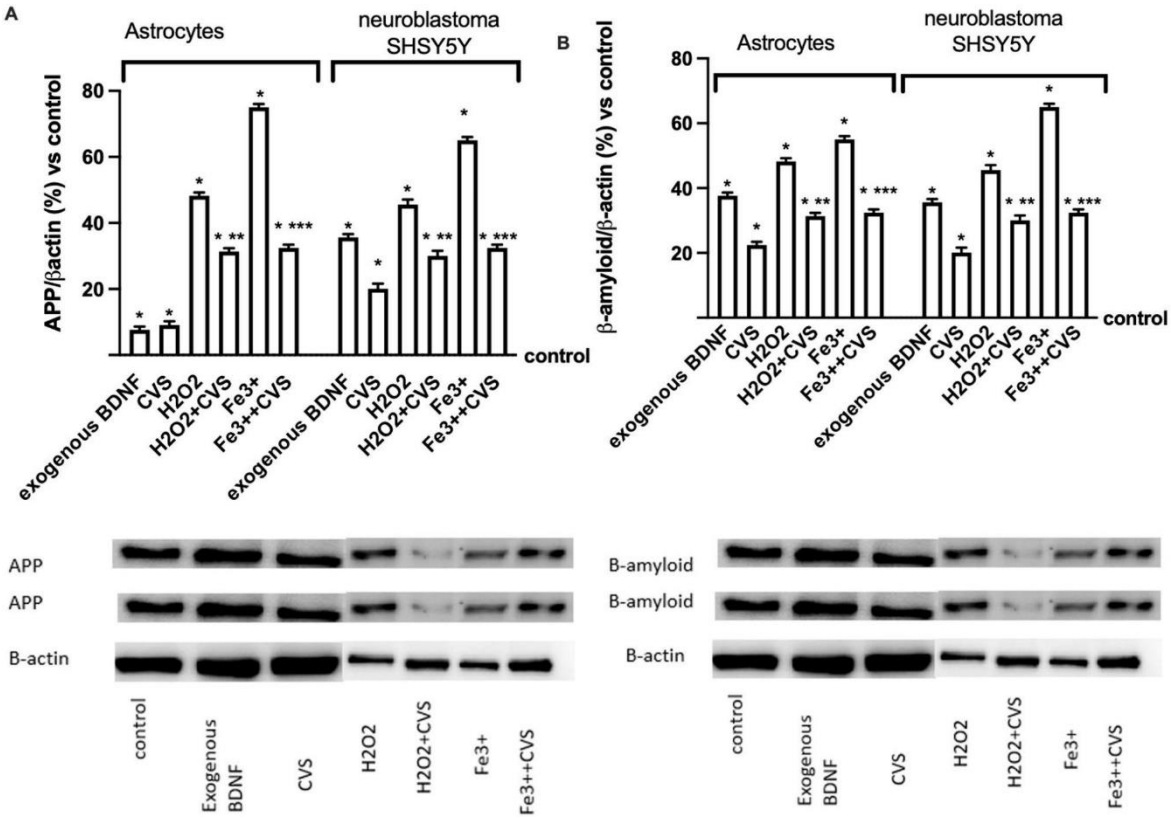

Figure 33. Western blot and densitometric analysis of (A) APP protein, and (B) β-amyloid in astrocytes and SH-SY5Y cell line. The abbreviations are the same as reported in Figure 27. Data represented are expressed as means ± SD (%) vs. control (0% line) of five independent experiments, each performed in triplicate. *$p < 0.05$ vs control, **$p < 0.05$ vs H_2O_2, ***$p < 0.05$ vs Fe^{3+}.

Finally, the expression of both pTau and SIRT-1 were analyzed in order to confirm the beneficial effect of CVS to slow down brain aging. As demonstrated in figure 34, the analysis of pTau in the presence of endogenously induced BDNF in astrocytes shows a low expression (about 8.8% compared to control), however, this expression was lower in SH-SY5Y (Figure 34) compared to control (about 7.9%, $p < 0.05$) and compared to exogenous BDNF (16.75%, $p < 0.05$). In addition, CVS confirms the beneficial effects exerted in the presence of induced BDNF in both astrocytes and SH-SY5Y (about 29.5% and 18.9% respectively compared to control), significantly improving SIRT1 expression ($p < 0.05$). All these findings support the hypothesis that CVS could be a suitable source for endogenous BDNF induction to potentially slow down degeneration and protect the brain during time. Moreover, these findings require to be performed on longer time periods of treatment to explore long term effects in order to suggest such protocol as adjuvant therapy in neuroprotection.

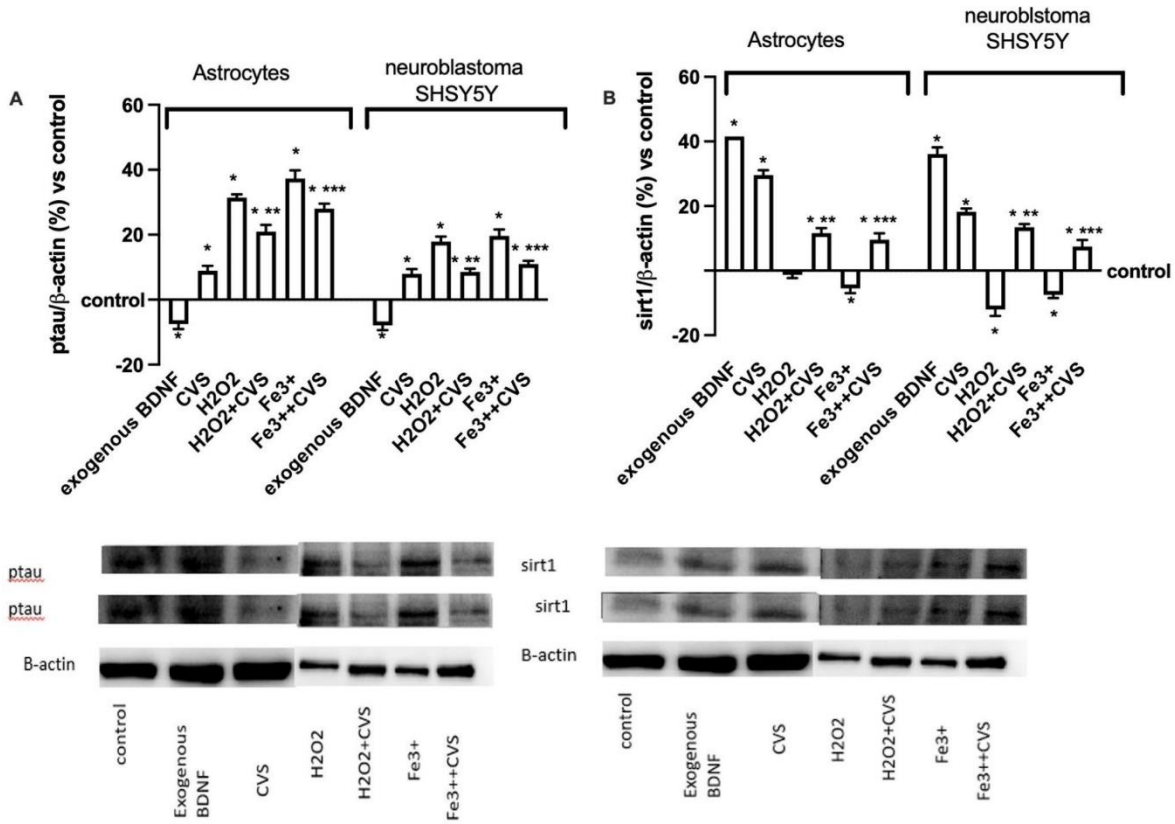

Figure 34. Western blot and densitometric analysis of (A) pTau protein, and (B) SIRT-1 in both astrocytes and SH-SY5Y cell line. The abbreviations are the same as reported in Figure 27. Data represented are expressed as means ± SD (%) vs. control (0% line) of five independent experiments, each performed in triplicate. *p < 0.05 vs control, **p < 0.05 vs H_2O_2, ***p < 0.05 vs Fe^{3+}.

5. Discussion

Based on the major theory of the aging process, oxidative stress has a crucial role in the genesis and progression of age-related illnesses in several tissues and organs. In this context the application of several bioactive compounds and/or natural extracts with antioxidant and anti-inflammatory properties will be promising dietary supplements applying to humans.

Project 1: Muscle aging

Multiple lines of research explored topics directly related to nutrients that further refined information on evidence-based nutrition recommendations to support physical activity [421]. Use of dietary supplements is widespread in many populations, including athletes, the elderly, sedentary people, and people with chronic diseases often on an empirical basis and without evidence based on experimental data [422; 423]. For this reason, there is growing interest in research of nutrients/nutraceuticals and associated mechanisms of action concerning skeletal muscle health with the aim of defining evidence-based recommendations. The supplements commonly used for the maintenance of skeletal muscle health are usually made up of proteins and amino acids (e.g., leucine, creatinine, and carnitine), associated with vitamins (e.g., vitamin D or vitamin C) or minerals (e.g., magnesium. or potassium), essential for many metabolic processes [421]. Recently, however, the need has arisen to develop new types of supplements capable of counteracting muscle damage related to hypercontractility during exercise. For this reason, in this work, the effectiveness of a combination of four substances, magnesium, potassium citrate, vitamin D3, and curcumin (MKVC) has been tested on the biological aspects related to a state of hypercontraction during physical exercise and which can lead to muscle damage. The results of this study show that the combination of these substances exerts a positive effect on myoblasts through the activation of intracellular mechanisms capable of stabilizing the beneficial effects of the individual agents on mitochondrial health. In particular, this effect was observed by the cell viability analysis which can rule out any cytotoxic effect or mitochondrial imbalance in the absence of limited respiration, which otherwise leads to a concomitant high rate of cell apoptosis.

In addition, our results support the hypothesis that MKVC can promote physiological differentiation in C2C12 cells as demonstrated by the analysis of cyclin D1, which is abundant in proliferating myoblasts, and confirmed by the analysis of desmin.

Furthermore, MKVC shows a significant impact on mitochondrial activity and on its membrane potential because it is capable of increasing ATP production and oxygen consumption. This is a crucial point for the proliferation of myoblasts. Some foods naturally contain a class of nutrients that have beneficial effects on health. These are called nutraceuticals. Skeletal muscle can also benefit from the combination of micro-, macronutrients, and nutraceutical substances both in terms of normal physiological trophism and in terms of response to physical exercise. Consequently, some studies have investigated the potential cooperative effect of combined nutritional "cocktails" [424]. One of the main areas of interest driving research towards the development of new supplements for the muscle is their possible role in controlling muscle cramps, typical sudden, involuntary, painful, and palpable muscle contractions that last from seconds to minutes. Muscle cramps can be associated with pathological conditions, but most commonly occur in the absence of known conditions [424; 425]. For this reason, some combinations of micronutrients, such as vitamins and minerals, are being studied to try to reverse this condition. This study fits into this context, in which the importance of the mechanism underlying the muscle contraction-relaxation cycle was further investigated. The time-course study performed on C2C12 cells to analyze Ca^{2+} and Mg^{2+} fluxes confirmed the importance of this mechanism in the genesis of cramps, indicating that MKVC better modulates a state of hypercontraction, restoring ion fluxes to more physiological values and remodeling the contractile phase with less cell fatigue in an in vitro model. Another important element in the balance between contraction and relaxation is the maintenance of a physiological value of inflammatory cytokines. MKVC can maintain the level of TNFα indicating that it could have a great effect in hypercontractility conditions. Furthermore, the results of this work showed that MKVC induces less intracellular accumulation of lactate, resulting in increased glucose consumption to support aerobic activity in C2C12 cells. These data suggest that glucose concentration and glycogen accumulation are consumed under physiological conditions after MKVC stimulation. Consequently, activation of PI3K/AKT and p38/MAPK confirmed our hypothesis, revealing the role of MKVC in assisting the energy conservation process in C2C12 cells. In particular, the mechanism of action of AMPK demonstrated the efficacy of MKVC in promoting glucose uptake, according to the classic activation mechanisms, which include mitochondrial modulation. Furthermore, the evaluation of JNK ½ and SMA showed that MKVC supports better maintenance of muscle cell survival systems, assuming greater efficiency even after contractile damage. The stimulation with caffeine was aimed at mimicking a state of hypercontraction which during exercise can lead to the onset of muscle cramps. Caffeine is a potent metabolic stimulant in skeletal muscle that has ergogenic effects

and has been shown to stimulate several exercise-like effects within skeletal muscle cells [426]. In vitro skeletal muscle treatment with caffeine promotes insulin-independent glucose transport, fatty acid oxidation, the release of Ca^{2+} from the SR, and mitochondrial biogenesis which is the basis of the contraction skeletal muscle. Caffeine treatment has been applied to skeletal muscle cells that have Ca^{2+} ionophore, with the specific intent of obtaining an exercise-like treatment, mimicking exercise activation and trigger Ca^{2+} changes like those found in exercise, mimicking the typical activation of exercise or exercise signals and trigger Ca^{2+} changes similar to those in exercise. For this reason, caffeine has been a widely accepted experimental model for examining exercise regulating changes in signal transduction and metabolism in skeletal muscle cells [427]. Our results revealed that MKVC is able to counteract the negative effect of caffeine, suggesting a long-term effect on muscle function restoration and complete post-trauma regeneration in an in vitro model. MKVC treatment, with or without caffeine pre-stimulation, supports the hypothesis that a new supplement based on the MKCV combination may have numerous roles during cell muscle activity and reveals beneficial effects on the biology of the muscle cells in the strong state contraction. Since some criticisms have been raised for the stimulation of caffeine additional experiments will be necessary in order confirm the present findings on in vivo model, which may be a pre-clinical option that precede MKVC human use.

Project 2: Retinae aging

Glaucoma includes a group of eye disorders that can lead to progressive and irreversible blindness [428]. Glaucoma damage is generally caused by an increase in intraocular pressure, which is supported by other factors, such as oxidative stress, leading to a progressive degeneration of the retinal ganglion cells and the optic nerve [429]. Oxidative stress plays a crucial role in the pathogenesis of age-related eye diseases, leading to the production of inflammatory cytokines, angiogenesis, proteins, and DNA damage, and, ultimately, apoptosis. Dietary supplementation of plant natural products has demonstrated preventive and therapeutic effects, based on their capacity to scavenge free radicals, and reduce enzymes involved in ROS production. It neutralizes the oxidative reaction that occurs in photoreceptor cells and upregulates the antioxidant defense system.

In particular, some extracts of herbal origin have also shown their capacity to reduce the opacification of the lens and the apoptosis of retinal cells and are able to inhibit the inflammatory markers of the blood-retinal barrier and improve ocular blood flow [430].

Deficiencies of specific nutrients have been found in patients with glaucoma, and dietary supplementation may play an important role in treatment [195]. Indeed, the goal of any strategy to prevent or modulate the degenerative cascade could be to act in the initial phase of the disease. Based on this, innovative strategies are further intensively investigated, to explore an innovative treatment of glaucoma that is capable of reducing the loss of functional retinal pigment cells in order to maintain patients' quality of life [431-434]. Recent studies show the importance of integrative therapy in patients at risk or with glaucoma [435; 436]. Mimicking human diseases in animal models in species, such as mice and bovines, has been a key resource to understand the pathogenesis of human eye diseases, and to develop novel therapeutic and drug delivery strategies [437]. Recently, the use of in vitro models for the study of glaucoma has opened up the possibility of studying the cellular and molecular mechanisms, which can help clarify the onset and progression of this disease [438].

In particular, the main models used in this research field include a wide variety of cell cultures, from cell lines to more complex models, such as tissue cultures (retinal organotypic cultures) and ex vivo preparations (in vitro preparations of ocular tissues) [438]. Furthermore, recent advances in regenerative medicine have led to the generation of 3D organic tissues (organoids) as organ-like structures to simulate a complex biological system. The development of retinal organoids is highly promising in regenerative medicine; organoids can be expanded and differentiated in vitro from ESCs and iPSCs [439-441]. If comprehensive, safe, and efficient protocols for the handling of organoids are yet to be developed, RGC cells have confirmed their key role in the glaucoma condition [438]. In this context, the efficacy of new nutraceutical compounds was tested on both in vitro (RGC cell) and ex vivo models (eyecup preparation). The combination of vitamin D3, gastrodin, lycopene, vitamin C, and blackcurrant (VGLCR) exerts a synergistic effect on retinal ganglion cells, indicating a possible new strategy to act in the early stage of glaucoma. Furthermore, reactive oxygen species have been implicated in the pathogenesis of various eye diseases, including mitochondrial dysfunction, which plays a crucial role in RGC cell degeneration; in this context, VGLCR is able to work on mitochondrial balance, supporting the hypothesis that this combination can be used to prevent cell loss. Since the main theory behind glaucoma concerns oxidative stress and IOP, further experiments were carried out in experimental models mimicking glaucomatous conditions by NMDA and H_2O_2. The pre- and post-treatment with VGLCR improved cell viability and reduced ROS production, thanks to the beneficial effects of VGLCR with a direct action on the activity of retinal cells by inhibiting cell loss. The data collected show that VGLCR is able to restore glaucoma-related damage caused by specific

inductors (such as NMDA and H_2O_2) by promoting cell survival. Furthermore, the specific intracellular pathway activated by VGLCR during the glaucoma condition was analyzed, starting from the activity of p53.

Our results show that there is a reduction in p53 activity following VGLCR treatment and this effect is related to the reduction of nuclear compartment damage and apoptosis. Furthermore, since oxidative stress is notoriously involved in cell apoptosis, iNOS analysis was performed to confirm that its modulation can prevent cell death. The pre- and post- administration of VGLCR improved cell survival, by reducing iNOS expression caused by the glaucoma inductors. In addition, the analysis of MMP9 demonstrated the ability of VGLCR to restore the alteration of the composition of the extracellular matrix caused by glaucoma, both in RGC and in eyecup preparations. According to the literature, structural changes are believed to anticipate functional loss, involving alterations in transcription factors and extracellular signaling pathways, and inflammatory cytokines. NDMA-induced retinal toxicity involves both JNK and p38 MAPK, with the inhibitors of each being foundas protective in this study. Similarly, JNK inhibition was protective against RGC loss in another ocular hypertensive model in a dose-dependent manner [442]. As reported above, the presence of VGLCR is able to significantly activate p38/MAPK and JNK, favoring the survival of RGC cells and eye tissues when added both before and after injury. This demonstrates its ability to prevent and/or restore the damage induced by glaucoma and confirms that inhibitionof p38 MAPK signaling in the retina could represent a therapeutic target to prevent the early stages of the disease. In this context, some further experiments were performed to verify the effectiveness of VGLCR directly on complex systems, such as explants of retinal tissues. Since elevated IOP is a major risk factor, animal models relevant for glaucoma include culture of RGC and optic nerve experimental damage induced by ocular hypertension [438]. Indeed, many of the animal models of glaucoma presented an elevated IOP by reducing the outflow ofthe aqueous humor [443; 444]. The opportunity to study using whole tissue cultures has clear advantages compared to a monolayer of cultured cells, allowing the study of cell-to-cell interactions and offering the possibility to maintain an anatomical structure under glaucoma conditions [445]. Thus, to understand the pathological changes underlying this disease, we have set up an ex vivo model based on bovine retinas to analyze several mechanisms activated by NMDA and H_2O_2 [390; 446].

Due to the high number of similarities between bovine and human systems as well as their retinal structure, these retinae appear to be a very promising alternative to animal experiments in ophthalmologic research [447]. Another important advantage of the use of bovine model is that in contrast to conventional cell culture models, the retina itself can be cultivated for some time. The culture of retinal tissue allows the maintenance of interactions and connections between neurons within the retina, and, for this reason, this model is very suitable for the screening of new therapeutic approaches [363]. Bovine eyecup preparation is a widely accepted model to reproduce the anterior chamber of the eye of glaucoma patients. This model is able to sustain a high pressure to assess some cellular responses [438].

Based on the advantages described above, we decided to also test the effects of VGLCR in this ex vivo model. In our experiments, it was possible to measure a significantly higher retinal mortality rate after NMDA and H_2O_2 treatment, while VGLCR administered both before and after inducing the glaucomatous injury was able to prevent retinal degradation. The beneficial role of this combination was also confirmed by the reduction of p53, exerted by the administration of VGLCR. Useful strategies could be to enhance the production of antioxidant enzymes, reduce ROS, or promote cytoprotective signaling pathways. With its antioxidant properties, this formulation could be used as a complementary therapy with a preventive effect on ROS production [448]. Different studies provide cumulating evidence, which supports the association of ROS with different aspects of the neurodegenerative process [186]. RGC cells are known to exhibit unique characteristics for their antioxidant defense mechanisms [449]. However, decreased ROS generation further promotes the survival of these cells [186]. Although currently available glaucoma therapy focuses on reducing IOP, some patients do not respond to this type of treatment, and research into RGC neuroprotection is emerging as a new therapeutic strategy. One of these strategies is precisely the reduction of oxidative stress [450]. Therefore, VGLCR demonstrates that it can restore glaucoma-induced oxidative damage by reducing the activity of iNOS and SOD. Furthermore, even if the presence of glaucoma caused damage to the retinal tissue, the response to the pre- and post-treatment with VGLCR can significantly activate the cell survival mechanisms in the early phase of the disease, by reducing the activation of p38, MAPK, and JNK. Indeed, these effects were mediated by the inhibitory effect induced by VGLCR on ocular oxidation (SIRT1) and ocular damage (OPA1).

Project 3: Brain aging

Today neurodegenerative disorders are considered chronic and incurable conditions, whose disabling effects can last for years or decades. This represents a huge burden of suffering for patients and costs for health organizations. Optimal cognitive functions are linked to an efficient neuronal plasticity and the ability of neurons or glial cells to improve the efficacy of the synapses through biochemical and morphological changes, both at a dendritic and axonal level [451]. However, as reported by many studies, this ability shows a marked age-related decrease [268]. At present, treatments available for these diseases are mostly symptomatic or palliative and include neurotransmitter modulators, hormonal therapies, anti-inflammatory drugs, deep brain stimulation and herbal products. Therefore, there is an urgent need to develop new solutions able to restore the physiological functions of the brain tissue. Moreover, one of the main problems concerning the administration of active ingredients into the central nervous system is the crossing of the blood–brain barrier. Modern drug delivery systems can consist both of biodegradable and non-biodegradable formulations, which offer advantages in terms of protection, absorption, penetration, and distribution of active ingredients. For this reason, the use of molecules already known for their exclusive functions within the brain and consequently physiologically predisposed to easily cross blood-brain barrier can be considered a valid option.

Recently, researchers' attention has focused on the involvement of the neurotrophic factors in the development of neuronal decay. Currently, it is common knowledge that there are three main neurotrophic factors: the brain-derived neurotrophic factor (BDNF), the nerve growth factor and the glial cell-derived neurotrophic factor [452]. BDNF in particular is associated with the modulation of neuroplasticity, which promotes the health of nervous tissue and also has the ability to counteract the effects of pro-inflammatory cytokines, which are key factors in neurodegenerative processes [453].

In recent years, the possibility of using a growth factor therapy has been hypothesized by exploiting the growing information that research has accumulated, mainly on BDNF. Indeed, BDNF seems to have a real therapeutic potential, based on the observation that in many disorders of the nervous system serum BDNF levels are altered [454]. However, a major problem is the delivery of the molecule to the affected cells. Although numerous studies have explored the possibility of administering BDNF through several approaches, such as gene therapy vectors, the development of mimetic peptides or even through direct administration into the nervous system, the results are still penalized by the lack of ease of use [454]. Attempts

to orally administer BDNF have so far yielded poor results due to the fact that BDNF is a moderately sized and charged protein and its transport through the intestinal barrier and BBB is not clear [454].

The key idea of this work is to use a low-dose BDNF solution to avoid the possible side effects of current therapies (such as sensitization and allergic reactions), supporting with experimental data a new potential therapeutic approach to treat or prevent neurodegenerative diseases. The concept of a low-dose is an important and innovative aspect and has been shown to be effective in many studies.

For example, in vivo treatment experiments with low-dose interferon were performed in many animal species [455]. This treatment has been shown to induce dramatic clinical improvement in models of both infectious and chronic inflammatory diseases [456].

This work demonstrated, in *in vitro* experimental models, that BDNF is able to cross both the intestinal and BBB barrier, thus demonstrating the safety of its use. This study also demonstrates for the first time the efficacy of low-dose BDNF SKA in counteracting oxidative damage, which is one of the mechanisms underlying age-related neurodegeneration. The possibility of administering BDNF in very small amounts is a great advantage, both for the low risk of adverse effects and for the lower cost of treatment.

In the design of the study, it was decided to use a particular solution preparation technique, which is called SKA. It has been hypothesized that the mechanism of bioactive molecules, such as hormones, neuropeptides and growth factors, subjected to SKA and administered at low dosage, consists in the sensitization or activation of some cellular (or plasma) receptor units by virtue of their high dilution, and practically in their physiological working in the order of micrograms for hormones [457] and picograms for the other messenger molecules [458]. The SKA method has been used in previous works, which showed that SKA solutions have better biological effects than corresponding solutions that did not receive the same treatment [394; 397; 459]. In the present study, it has been shown that BDNF SKA does not induce neuronal stress and it is able to counteract the formation of ROS.

During brain aging, the cells that show the first signs of degeneration are the astrocytes, despite the fact that subsequently the most important site of damage is represented by cortical neurons [460]. BDNF SKA is able to increase cell viability in both neuronal cells and astrocytes, representing an important resource for the health of the nervous system.

Furthermore, the aim of this study was to explore the timing of administration, to see if it was possible to identify an effective protocol that could be used in humans in the future. It can be hypothesized that the six-day protocol, consisting of a single administration followed by six

days of measurements, stimulates cells without overloading normal physiological regulation. In this way a greater crossing of BDNF is achieved through the BBB, also inducing a low concentration of ROS and a reduced neuronal stress. In this way the normal cellular physiological processes are activated.

The in vivo part of this research has allowed us to demonstrate that BDNF SKA has a high capacity to cross the enterohepatic circle and is able to remain in the bloodstream for at least 24 h. This makes the BDNF SKA a potential candidate for use as a food supplement. Since BDNF, necessary for the survival of neurons, is synthesized after the encoding of its specific gene, its expression was studied in this research. We observed that BDNF SKA at 24 h plus 24 h was able to induce the expression of endogenous BDNF, indicating a better effect of stimulation and induction of endogenous BDNF production. The beneficial effects of BDNF SKA have also been confirmed by the analysis of the amyloid protein precursor. Taken together, our results suggest that the simultaneous activation of at least ERK/MAPK is necessary to mediate a complete BDNF-dependent activation of the APP promoter [461].
APP protein plays a central role in the development of Alzheimer's disease, its expression, metabolism, splicing and secretion have been demonstrated to be regulated by ligands of the membrane tyrosine kinase receptors like BDNF [462]. Furthermore, the study of the intracellular pathways demonstrated a significant increase in the expression of ApoE, which is a member of the low-density lipoprotein receptor gene family, mainly produced by the astrocytes in the brain. ApoE has been identified as the receptor that mediates amyloid β (Aβ) uptake and clearance by astrocytes, thus increasing glial LDLR levels, which may promote Aβ degradation within the brain [463-465]; these data indicate a positive effect on brain trophism exerted by BDNF SKA and an increase in SIRT1 phosphorylation, confirming a potential role in counteracting the known mechanisms that lead to brain aging. Indeed, SIRT1 has recently been shown to play a role in normal cognitive function and synaptic plasticity, counteracting cognitive decline and neurodegenerative disease in aging [466; 467].
The effectiveness of BDNF SKA has been carefully observed, allowing the serum BDNF levels to be maintained for up to six days after a single administration; these data suggest that BDNF SKA is able to remain in the nervous tissue for a long time even in the absence of treatment, triggering its own physiological production by cells better than high-dose BDNF. All our results support the hypothesis that treatment with BDNF SKA can protect the brain over time by inducing a physiological mechanism capable of slowing cell degeneration and may be a possible therapeutic strategy for the elderly population, in order to improve cognitive function.

BDNF is also able to exert effects outside the nervous system, for example on the immune system [468]. Therefore, interactions between the immune system and the effects described in this work could be hypothesized. The lack of this part could be a weak point in our research, which will be filled in subsequent research. On the other hand, one of the strengths of this study is the confirmation of the efficacy and safety of a low-dose BDNF. The low-dose administration reduces the side effects of active molecules, without reducing their effectiveness is an important and innovative aspect. Indeed, the common clinical practice has long understood the essential importance of small stimuli compared to strong ones, which trigger self-regulatory and self-repairing mechanisms in the organism [469]. In brain aging there is a decline in normal antioxidant defense mechanisms leading to increased brain vulnerability and finally to the deleterious effects of oxidative damage [41]. Indeed, a large body of experimental research indicates that the brain is very susceptible to oxidative damage due to a high concentration of polyunsaturated fatty acids and transition metals that are involved in the generation of the hydroxyl radical [470; 471].

Members of the ROS family, if not properly detoxified, start the process of oxidative damage, which can be defined as a chain reaction leading to sequential damage of all cellular components, in particular of lipids and proteins [472]. Oxidative stress is considered one of the main mechanisms of cellular aging due to the ease of amplification of the damage and to the large number of target molecules [41].

Cognitive deficits are the most common consequences of the aging process, and they are characterized by massive neuronal loss, cognitive dysfunction, and memory loss. Their incidence and prevalence continuously increase with advancing age [473]. Indeed, it has been shown that low serum BDNF levels are linked to increased cognitive impairment [474]. Moreover, BDNF helps to protect neurons from damage caused by infection or injury [475] and participates in neuronal growth and maintenance and in different aspects of activity-dependent synaptic physiology by acting across different spatial and temporal domains [476]. However, because of the difficulties associated with the administration of exogenous proteins into the central nervous system (CNS), it is important to consider the possibility of using endogenous sources of BDNF, for example, inducing increased glial cell activity. It is well known that glial cells increase expression of a variety of growth factors, including BDNF. In particular, in the adult brain, astrocytes are the cells responsible for maintaining neuronal and synaptic function [477].

The importance of BDNF and its regulation to improve healthy aging is more evident and for this reason successive studies analyze the ability of several natural extracts to act directly on

these neurotrophin markers. Many studies have shown the effectiveness of nutraceuticals based on their antioxidant functions to reduce oxidative stress and restore cognitive functions preventing age-related diseases [478]. These dietary supplements contain the natural extracts with antioxidant activity which act through free radical scavenging the expression of inflammatory cytokines to induce neuroprotection [479]. These natural extracts such as curcumin, vitamin D3 and 6-shogaol have shown promising effects [418; 480]. Based on these findings, the combination of curcumin, vitamin D3, 6-shogaol (CVS) could provide protection against oxidative stress and neuroinflammation. In this context, human neuroblastoma SH-SY5Y cell line which is a widely used model to study neuronal health and primary astrocytes were treated with CVS to explore the possible use in humans after oral intake. Since the brain is highly susceptible to overproduction of ROS and becomes under oxidative stress as a trigger in brain aging and neurodegeneration, the efficiency of CVS to alleviate the oxidative stress or damage induced by H_2O_2 and Fe^{3+} to mimic brain aging conditions was verified. CVS is able to reduce ROS production due to their antioxidant activity and maintain mitochondrial balance to prevent neuronal loss. iNOS and SOD3 analysis confirms that; in particular, CVS can prevent cell death improving cell survival by reducing both iNOS and SOD3 expressions.

Both oxidative stress and iron-induced damage are implicated in several neurodegenerative diseases through affecting several hallmark proteins such as APP, β-amyloid, SIRT1 and pTau [481]; additional experiments also investigated their role in the presence of the CVS. CVS confirms its ability to preserve the brain function by the modulation on the expression of these proteins under all (oxidative stress and catalytic iron-damage) maintaining APP cleavage and trafficking, binding to Aβ peptide to prevent their aggregation, reducing hyperphosphorylated Tau protein and improving SIRT1 to provide neuroprotection. Since the concentration of serum BDNF in late adulthood was less than young people [482], several research studies have highlighted curcumin, vitamin D, and 6-shogaol as potential candidates for neuroprotection through endogenous upregulation of BDNF expression [346; 483; 484]. Therefore, CVS can provide further neuroprotection inducing BDNF expression under both physiological and aging mimicking conditions.

Astrocytes are mostly affected by oxidative stress due to their high mitochondrial metabolism activity and are highly implicated in neurodegenerative diseases as they can propagate their damage to neighboring neurons [485]. Moreover, astrocytes are treated with CVS to investigate the BDNF expression under physiological and brain aging mimicking conditions.

Data obtained showed that after 24 h, CVS is able to induce BDNF expression providing neuroprotection, neuroplasticity, and cognitive functions; CVS post treatment in brain aging mimicking conditions is able to revert the damage by endogenous production of BDNF. Since there is a crosstalk between astrocytes and neurons, the astrocyte-conditioned medium collected is used to stimulate neuronal cells to verify the mechanism of neuroprotection. Also in this case CVS is able to counteract the induced damage confirming its effectiveness to induce BDNF production and providing neuroprotection. In addition, this production is supported by the data about the expression of proBDNF and TrkB (BDNF receptor) along with brain aging and neurodegeneration hallmarks such as APP, β-amyloid, pTau and Sirt1.

In particular, under H_2O_2 and Fe^{3+} conditions proBDNF production is reduced and TrkB is switched off; conversely such effect is reverted after CVS indicating its positive effect to stimulate BDNF expression. Treating SH-SY5Y cell line with astrocyte conditioned medium CVS is able to activate TrkB confirms the interaction between astrocytes and neurons to provide neuroprotection and neuronal survival [486].

Furthermore, CVS is also able to modulate APP, β-Amyloid, pTau and SIRT-1 expressions. In particular CVS counteracts the decrease in the expression of both APP and β-Amyloid on both conditions in astrocytes and in SH-SY5Y cells, also maintaining APP integrity and cleavage into β-Amyloid [487]. In addition, CVS's beneficial effect to slow down aging is determined by analyzing the expression of pTau and SIRT-1; CVS increased the expression of Sirt-1 due to providing an antioxidant action [466].

All of these findings support the hypotheses on the beneficial effects of CVS to slow down brain aging, acting like a natural inductor of BDNF to provide brain protection. These findings provide a preliminary data on the effects on the early stages in brain aging and neurodegeneration, however, these experiments require to be performed on longer time periods of treatments to explore the long-term effects and the effects at late stage of damage in order to suggest such protocol as adjuvant therapy for neuroprotection.

6. Future Perspectives

Because oxidative stress is intimately connected to aging, inflammation, and a range of degenerative illnesses, main research was being conducted on the development of synthetic antioxidants and their potential for combating atherosclerosis and other ROS-mediated degenerative disorders [488]. However, subsequent research has identified the natural antioxidant functions of several bioactive molecules and natural extracts, such as polyphenols and carotenoids, as well as their beneficial effects in lowering the risk of several chronic diseases, such as cancer, cardiovascular disease, and neurodegenerative disorders [489]. Recently, there has been a surge of interest in studying the synergistic effects of various natural extracts and bioactive molecules in order to propose them as preventive or therapeutic agents [490].

Furthermore, because reduced BDNF expression is critical in the pathophysiology of AD, some research has included directly injecting exogenous BDNF into the hippocampus [491]. However, fresh promising research utilizing natural extracts have demonstrated their ability to stimulate endogenous BDNF expression [492]

Because of advancements in experimental research, food industry companies have recently focused their attention on further introducing plant extracts, vitamins, and micronutrients as supplements into the market due to their high efficiency as protective and therapeutic agents, as these compounds possess high antioxidant and anti-inflammatory capacities for cellular protection against oxidative stress [493].

For these reasons, rather than using them as single agents to protect or alleviate aging or age-related disorders, researchers should investigate combining different bioactive molecules and natural extracts with antioxidant activity to create formulations with enhanced antioxidant and anti-inflammatory activity. Modifying the delivery mechanism of these supplements, such as employing nano-delivery systems, is also a potential option for increasing bioavailability and the efficacy of guiding the formulation into the proper tissue. In addition, alternative strategies for inducing neurotrophins endogenously or injecting exogenous BDNF with special preparations to provide the needed quantity and retain its capacity to cross the BBB in order to use them as dietary supplements or delivering them as adjuvant therapeutic alternatives.

7. References

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