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Red wine micronutrients as protective agents in Alzheimer-like induced insult

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Abstract

Plant extract micronutrients are commonly added to diets for health and prevention of degenerative disease. However, there are barriers to the introduction of these products as antioxidant therapies in counteracting chronic human diseases, probably because the molecular bases of their therapeutic potential are poorly clarified. The present study was designed to evaluate the possible protective effect of combined micronutrients present in black grape skin on toxicity induced by 25–35 β-amyloid peptide or by serum of Alzheimer's disease patients, in human umbilical vein endothelial cells (HUVECs).

The hypothesis was tested by examining the results of lactic dehydrogenase (LDH) release to estimate cytoplasmic membrane breakdown; activity of mitochondrial complexes, reactive oxygen species (ROS) production and malonyl dialdehyde (MDA) levels as markers of oxidative stress induction and COMET assay to evaluate DNA fragmentation. The results demonstrate that black grape skin extract reduces the ROS production, protects the cellular membrane from oxidative damage, and consequently prevents DNA fragmentation. The experimental results suggest that this natural compound may be used to ameliorate the progression of pathology in AD disease therapy.

Keywords: Alzheimer's disease; Human umbilical vein endothelial cells (HUVECs); 25–35 β-amyloid peptide; Grape skin; Oxidative stress

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder associated with aging, characterized by selective neuronal loss in discrete regions of the central nervous system. The major microscopic alterations in AD are senile plaques (SPs) (also known as dense-core or neuritic plaque) and neurofibrillary tangles (NFT) formation, selective neuron loss and shrinkage, synapse loss, and amyloid angiopathy [19]. NFT and SPs represent an accumulation of intraneuronal and extracellular filamentous protein aggregates. Hyperphosphorylated tau is the major protein in NFT; amyloid β peptidee (Aβ), derived from the amyloidal transmembrane precursor protein (APP) through cleavage by secretase, is the major protein in SPs and amyloidal angiopathy [10]. The pathophysiology of the disease is complex and probably involves multiple overlapping and redundant pathways of neuronal damage. However the primary cause of AD remains unknown. Several lines of evidence suggest the involvement of oxidative stress in the pathogenesis of AD [21] with abnormalities observed in neurons and glial cells as well as in fibroblasts, platelets and vascular cells. In particular, there is increasing evidence that accumulation of Aβ, especially in its aggregate form, exacerbates free radical production causing cytotoxic events. The Aβ aggregation process is accelerated by transition metals via metal-catalyzed oxidation of Aβ peptidee [7,9,11]. In particular, it has been shown that Aβ peptide produces hydrogen peroxide (H_2O_2) through metal ion reduction, with concomitant release of thiobarbituric acid-reactive substances (TBARS), a process probably mediated by formation of hydroxyl radicals [11]. The increase in free radicals results in protein carboxyl formation and lipid peroxidation producing alteration of cellular homeostasis and cell functions [11,43]. Antioxidants such as vitamin E, estrogens or melatonin have demonstrated neuroprotective effects on Aβ-mediated cytotoxycity [43]. Apart from Aβ aggregation, many other processes may induce oxidative stress and cell death in AD, such as the release of cytokines, prooxidants [25], energy deprivation from impaired glucose utilization, defects of oxidative metabolism or loss of trophic factor stimulation [2,14]. The convergence of multifactorial aetiology of AD in a common final pathway involving free radicals and pathological oxidation, raises the possibility of the therapeutic use of antioxidants in delaying its development. Increasing evidence, over the last few years, indicates the protective effect of vegetables, fruits, and medicinal herbs in the prevention of free-radical mediated diseases [27,34]. Supplementation of diets with plant extracts for prevention of degenerative diseases is popular but the molecular basis of their therapeutic potential is poorly clarified. Among the different micronutrients, hydroxystilbene resveratrol (a plant constituent present in numerous foods, particularly the skin of grapes) may be considered one of the most promising molecules in protecting against neurological diseases such as AD and Parkinson. It shows several biological and pharmacological activities [1,5,33], and is a potent and effective inhibitor of the formation of fibrils, abnormal structures around nerves that are a hallmark of AD.

Studies of Thomas et al. [40] on vascular endothelial cells have demonstrated that Aβ is able to produce an excess of superoxide radicals which alter endothelial structure and function. These alterations in vascular tone and endothelial damage were prevented by superoxide dismutase (SOD), suggesting that Aβ may play a crucial role in mediating free radical elicited vascular abnormalities and subsequent neurodegeneration effects. Alterations in the functions of the blood–brain barrier and the brain parenchyma have been reported to be common in AD patients [16]. Endothelial cells, in fact, serving as interface between the circulating blood and the vascular wall or surrounding tissues, participate in several pathological events such as the immune response, inflammation, thrombosis, arterosclerosis,

tumor growth and also in the pathology of AD. The aim of our work was to test a crude extract of black grape skin as protective compound against toxicity induced in human umbilical vein endothelial cells (HUVECs) either by β 25–35 amyloid peptide or by serum of AD patients, known to contain a natriuretic apoptotic factor [22].

Methods

Drugs and chemicals

Crude extract of black grape skin employed in this study was a kind gift of GEYMONAT (Res Vin® Frosinone, Italy). The content of one capsule was dissolved daily in 10 ml of 30% ethanol in PBS and 50 μl was added to each dish containing approximately 7×10^5 cells (30 μg/ml). Under these conditions ethanol was not toxic, nor did it affect the action of the different treatments.

25–35 β-amyloid peptide and heparin were obtained from Sigma Aldrich Co. (St. Louis, USA). β-Nicotinamide-adenine dinucleotide (NADH) were obtained from Boehringer Mannheim GmbH (Germany). All other chemicals were purchased from GIBCO BRL Life Technologies (Grand Island, NY, USA).

Human umbilical vein endothelial cells (HUVECs)

HUVECs were obtained from pooled human umbilical cords from healthy women who underwent uncomplicated term pregnancies, as described elsewhere [15]. Cells were grown in gelatin coated plastic in medium M199 supplemented with endothelial cell growth supplement (ECGS; 20 μg/ml), heparin (1625 U.I./ml) and 20% fetal calf serum (FCS). The cultures were maintained for 12 days at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 and the medium was changed every three days; in the cells treated with serum of Alzheimer's disease patients or with serum from healthy elderly subjects, the last change of medium was carried out with serum-free medium.

Treatments

HUVECs (1×10^6 cells/dish), 12 days after seeding were differently treated with: a) β 25–35 amyloid peptide (5 and 20 μg/ml); b) human serum activated and inactivated (56 °C for 15 min) from Alzheimer's disease patients (10%); serum from healthy elderly subjects was used as controls. In the two different treated groups of cells the effect of crude extract from grape skin (30 μg/ml) was examined. After 24 h from the time of treatments the cells were immediately analysed.

Lactic dehydrogenase (LDH) release

Lactic dehydrogenase (LDH) activity was spectrophotometrically measured, using an equivalent number of cells (1×10^6 cells/dish), in the culture medium and in the cellular lysates at $\lambda = 340$ nm by analysing the NADH reduction during the pyruvate–lactate transformation [26]. The percentage of

released LDH was calculated as percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that in the culture medium. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

Mitochondrial enzymatic activities

The mitochondria were obtained according to the following procedure. HUVECs, pooled (3–4 dishes) to obtain approximately 4×10^6 cells, were mechanically harvested in PBS and centrifuged at 800 *g* for 10 min, and the pellet was resuspended in 500 μl of isolation medium (0.3 M sucrose, 10 mM Tris, 0.5 mM EDTA, pH 7.4). The cells were then homogenized in the same medium and centrifuged at 1000 *g* for 10 min. The mitochondrial pellet obtained was washed in PBS, pH 7.4, solubilized in 100 μl of the same buffer, and sonicated.

Mitochondrial respiratory chain enzymatic activities were determined spectrophotometrically. Complex I (NADH: ubiquinone 1-reductase, EC 1.6.5.3) was measured as the rotenone-sensitive rate of NADH oxidation according to Ragan et al. [32]; complex II–III (succinate: cytocrome-*c* reductase EC 1.8.3.1) according to King [20]; complex IV (ferrocytocrome-*c*: oxygen oxidoreductase EC 1.9.3.1) according to Wharton and Tzagoloff [42].

ATPase activity was evaluated as inorganic phosphate obtained by ATPase-catalyzed ATP demolition in the presence of oligomycin. Double-distilled water (440 μl) and 500 μl of 100 mM ATP (pH 6.5–6.8) were added to 10 μl of mitochondrial suspension. This suspension was incubated at 30 °C for 10 min and the enzymatic reaction was stopped by adding 10 μl of 10% SDS (solution A). "Reactive mix" (800 μl) comprised: 0.5 M malachite green, 4.2% $(NH_4)_2MoO_4$ in 4 M HCL, 160 μl Triton X-100, 100 μl of solution A. After 5 min, to allow for colour stabilization, the samples were analysed with a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) at $\lambda = 660$ nm. The values were calculated as nmoles per mg of protein and were expressed as percentage of the control. Protein concentration was measured according to Bradford [4].

Reactive oxygen species (ROS) assay

Reactive oxygen species (ROS) formation was estimated directly in the dish (1×10^6 cells) using a fluorescent probe, 2′–7′-dichlorofluorescein diacetate (DCFH-DA), according to Hunot et al. [12]. DCFH-DA (5 μM) was added to monolayer HUVECs (control and differently treated) for 20 min and in a humidified atmosphere (5%CO_2 and 95% air) at 37 °C. Dye-loaded cells were washed with PBS and the fluorescence of oxidized DCF, corresponding to the level of ROS production, was observed directly in the dishes by fluorescence microscope (Leika, Wetzlar, Germany): excitation 488 nm, emission 525 nm.

Malonyl dialdehyde (MDA) analysis

Malonyl dialdehyde (MDA) in cell suspension (1×10^6 cells) (20 mM potassium buffer, pH 7.4) was measured by monitoring the formation of thiobarbituric acid-reactive substances (TBARS) using the method of Chanvitayapongs et al. [6]. Briefly, 2 ml of each sample treated with trichloroacetic acid (15% w/v) containing 1 mM EDTA was centrifuged at 1000 *g* for 10 min. The supernatant was heated at 100 °C with an equal volume of thiobarbituric acid (TBA) (0.7% w/v) for 20 min and,

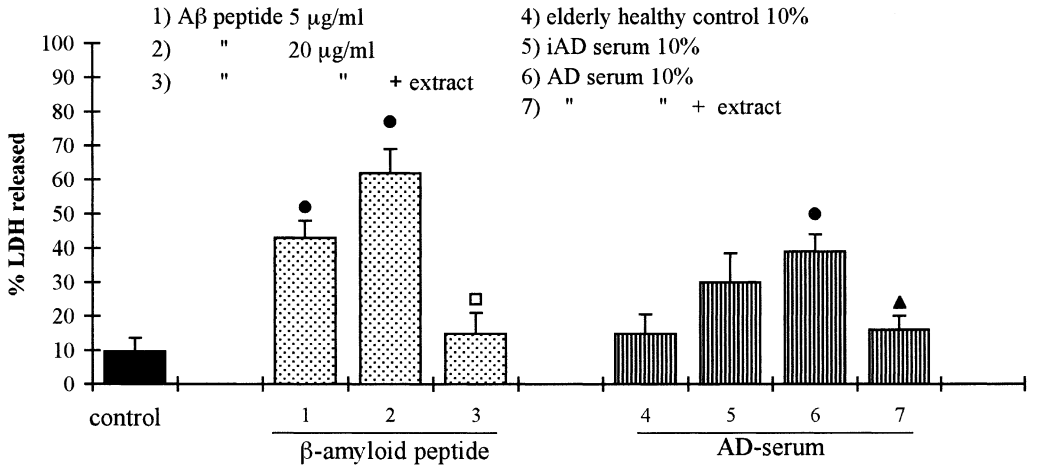

Fig. 1. Percentage of lactate dehydrogenase (LDH) activity released in the medium of untreated and treated HUVECs. The values are the mean ± SEM (n = 8). ●Significant vs. control untreated cells, ○significant vs. elderly healthy control, □significant vs. 20 μg/ml Aβ peptide, ▲significant vs. 10% AD serum; $p < 0.01$ is referred to all statistical analysis.

after cooling, the absorbance at λ = 532 nm was measured. Values were expressed as nmoles of malonyl dialdehyde formed per mg of protein. Protein concentration was measured according to Bradford [4].

Table 1
Mitochondrial enzymatic activities measured in untreated and treated HUVECs

Treatment		NCCR (Complex I)	SCCR (Complex II–III)	COX (Complex IV)	ATPase
		% of control			
Control	100				
β-amyloid peptide 5 μg/ml		90.0 ± 0.6*	85.1 ± 1.5*	81.3 ± 1.5*	95.1 ± 1.4*
β-amyloid peptide 20 μg/ml		80.3 ± 3.2*	73.3 ± 3.1*	70.2 ± 1.6*	89.0 ± 0.5*
β-amyloid peptide 20 μg/ml+ extract		92.5 ± 1.2[a]	97.0 ± 1.9[a]	89.0 ± 2.3[a]	96.1 ± 2.6[a]
Elderly healthy control 10%	100				
Inactivated AD serum 10%		100 ± 1.1	89.1 ± 2.2[b]	88.0 ± 3.4[b]	95.1 ± 1.8[b]
Activated AD serum 10%		80.1 ± 1.3[b]	68.3 ± 2.5[b]	64.1 ± 2.5[b]	76.3 ± 2.3[b]
Activated AD serum 10% + extract		91.0 ± 0.7[c]	88.1 ± 0.8[c]	91.0 ± 1.2[c]	95.0 ± 0.9[c]

Values are the mean ± SEM (n = 8).
$p < 0.01$ is referred to all statistical analysis.
* Significant vs control untreated cells.
[a] Significant vs 20 μg/ml Aβ.
[b] Significant vs elderly healthy control.
[c] Significant vs 10% AD serum.

DNA analysis by COMET assay

The presence of DNA fragmentation was examined by single cell gel electrophoresis (COMET assay), according to Singh et al. [37]. Briefly, $0.8-1 \times 10^5$ cells were mixed with 75 μl of 0.5% low melting agarose (LMA) and spotted on slides. The "minigels" were maintained in lysis solution (1% *N*-laurosil-sarcosine, 2.5 mM NaCl, 100 mM Na_2EDTA, 1% Triton X-100, 10% DMSO, pH 10) for 1 h at 4 °C, and then denatured in a high pH buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) for 20 min, and finally electrophoresed in the same buffer at 18 V for 45 min. At the end of the run, the "minigels" were neutralised in 0.4 M Tris–HCl, pH 7.5, stained with 100 μl of ethidium bromide (2 μg/ml) for 10 min and scored using a Leika fluorescence microscope (Leika, Wetzlar, Germany) interfaced with a computer. Software (Cayman Sarin, Florence, Italy) allowed us to analyse and quantify DNA damage by measuring: a) tail length (TL), intensity (TI) and area (TA); b) head length (HL), intensity (HI) and area (HA). These parameters are used by the software to determine the level of DNA damage as: i) the percentage of fragmented DNA (TDNA), and ii) tail moment (TMOM) expressed as the product of TD (distance between head and tail) and TDNA.

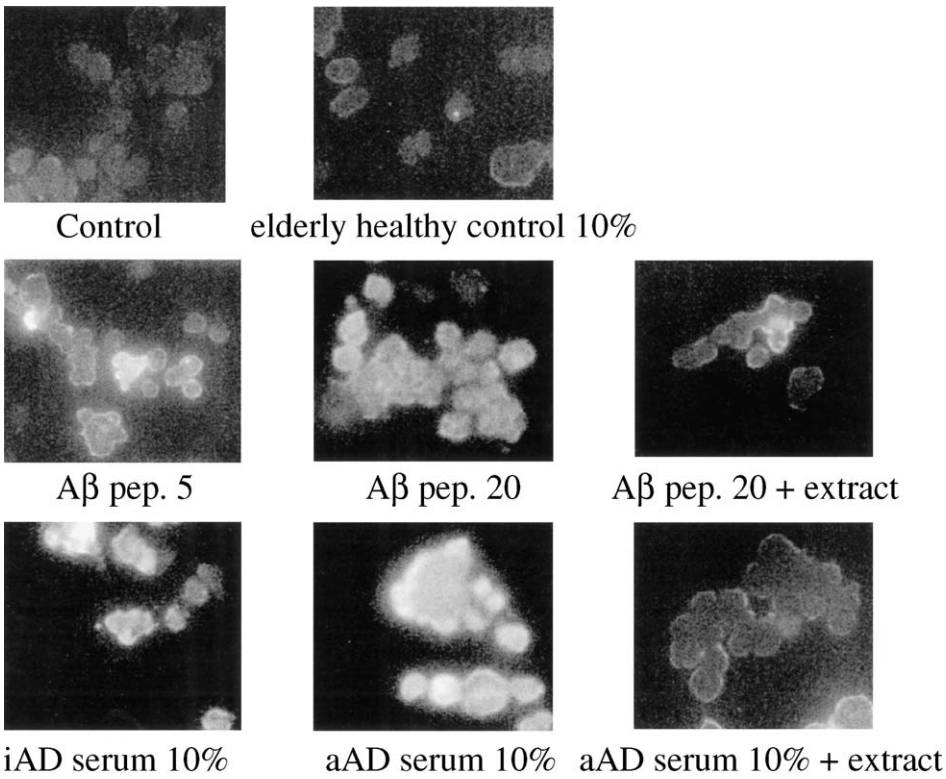

Fig. 2. Qualitative ROS formation evidenced by DCFH-DA in untreated and treated HUVECs. The probe was added to the cultures 30 min before the end of the treatment. The photomicrographs obtained by fluorescent microscopy are representative of one experiment. Aβ pep. 5: 5 μg/ml Aβ peptide; Aβ pep. 20: 20 μg/ml Aβ peptide; iAD serum 10%: 10% inactivated AD serum; aAD serum 10%: 10% activated AD serum; The analysis was performed four times in duplicate.

Statistics

Means ± SEM are given. Statistical analysis between various experimental results was performed using Student's *t–test*.

Results

The results first of all evidence the breakage of cellular membrane elicited by the different treatments valued as percentage of LDH release (Fig. 1). In the different treatments, LDH release was fully prevented by black grape skin extract.

The levels of mitochondrial enzymatic activities are reported in Table 1. β 25–35 amyloid peptide (20 μg/ml) induced a decrease in activity of NCCR, SCCR, COX and ATPasi. Also active AD serum (10%), added to serum free culture, induced a decrease in all enzymatic activity with respect to control values, the II/III (SCCR) and IV (COX) complexes being particularly affected. The treatment with the black grape skin extract restored the normal values in cell cultures exposed to the stressing agents.

To verify the relationship between mitochondrial activity dysfunction and oxidative stress, the ROS production and MDA levels were examined, as reported in Fig. 2 and Table 2. Treatment with β 25–35 amyloid peptide (20 μg/ml) or the presence of 10% AD serum induced a ROS production. In each condition, the production of ROS was proportional to the increase in thiobarbituric acid reactive substances (TBARS) (Table 2). In the presence of black grape skin extract, cell conditions were the same as controls.

The results on genomic DNA, analysed by COMET assay alkaline electrophoresis are presented in Table 3. The data of TDNA and TMOM clearly evidence a DNA damage in cells exposed to 20 μg/ml β

Table 2
MDA (malonyl dialdeyde) content in control and treated HUVECs measured by monitoring the formation of thiobarbituric acid reactive substances (TBARS)

Treatment	MDA nmoles/mg prot.
Control	1.95 ± 0.4
β-amyloid peptide 5 μg/ml	2.94 ± 0.3*
β-amyloid peptide 20 μg/ml	3.52 ± 0.4*
β-amyloid peptide 20 μg/ml + extract	2.18 ± 0.3[a]
Elderly healthy control 10%	2.23 ± 0.2
Inactivated AD serum 10%	2.90 ± 0.4[b]
Activated AD serum 10%	3.26 ± 0.3[b]
Activated AD serum 10% + extract	1.97 ± 0.2[c]

Values are the mean ± SEM (n = 8).
$p < 0.01$ is referred to all statistical analysis.
* Significant vs control untreated cells.
[a] Significant vs 20 μg/ml Aβ.
[b] Significant vs elderly healthy control.
[c] Significant vs 10% AD serum.

Table 3
Comet assay of genomic DNA of untreated and treated HUVECs

Treatment	TDNA	TMOM
Control	21 ± 2	31.5 ± 3
β-amyloid peptide 5 μg/ml	51 ± 4*	408 ± 40*
β-amyloid peptide 20 μg/ml	86 ± 7*	1118 ± 25*
β-amyloid peptide 20 μg/ml + extract	26 ± 2[a]	65 ± 9[a]
Elderly healthy control 10%	23 ± 1.8	34.2 ± 4.5
Inactivated AD serum 10%	42 ± 4[b]	289.8 ± 10[b]
Activated AD serum 10%	49 ± 5[b]	377.3 ± 54[b]
Activated AD serum 10% + extract	26 ± 2[c]	46.4 ± 3[c]

Values are the mean ± SEM (n = 8).
$p < 0.01$ is referred to all statistical analysis.
* Significant vs control untreated cells.
[a] Significant vs 20 μg/ml Aβ.
[b] Significant vs elderly healthy control.
[c] Significant vs 10% AD serum.

25–35 amyloid peptide and 10% AD serum. The addition of the natural compound under investigation, reduced the generation of ROS, preventing the DNA damage.

Discussion

Endothelial cells serve as interface between the circulating blood and the vascular wall or surrounding tissues and interact with various types of cells such as leukocytes, platelets and smooth muscle cells. They participate in several pathological events such as the immune response, inflammation, thrombosis, arterosclerosis, tumor growth and also in the pathology of AD. Alterations in the functions of the blood–brain barrier and the brain parenchyma have been reported to be common in AD patients [16]. The amyloid deposition in the wall of vessels, in fact, causes degeneration of endothelial cells or obliteration of the vessel lumen [40].

The accumulation of senile plaques is an important hallmark of AD, but the mechanisms that can regulate Aβ deposit and its interaction with molecular "events" in activating cell death pathways are not well known. Both neuronal and endothelial origins of Aβ deposit have been postulated. Aβ has been shown to disrupt neuronal homeostasis, increase ROS production and cause oxidative damage to APP present in both neurons and glial cells [38]. In addition, experimental data indicates that Aβ interacts with vascular endothelial cells inducing, through ROS increase, structural and functional alterations [24].

In the present study we generate in vitro an AD-like condition, treating endothelial cells with β 25–35 amyloid peptide and alternatively with serum from AD patients, in which the presence of a natriuretic factor has been demonstrated [22]. Moreover, since antioxidants such as vitamin E and vitamin C have been used to ameliorate oxidative damage in AD, we have focused on an extract of black grape skin. Black grape skin is known to be very rich in micronutrients, such as quercetin and its glycoside rutin, two well known antioxidants, vitamins C and E, and high concentrations of resveratrol, all involved in ameliorating oxidative stress in both cytosol and membrane compartments in the cells [6].

In particular, resveratrol (trans-3,5,4′ -trihydroxystilbene), one of the major components of red wine, is a natural phytoalexin found in a wide variety of plant species, abundantly present in the seeds and skin of grapes [39]. Regular consumption of red wine has been correlated to the low incidence of atherosclerosis among the French (the so called ‘‘French paradox’’) [33], to a protective effect against certain neurological disorders and to a reduction in both the incidence of age-related macular degeneration [28] and Alzheimer’s disease [29]. In addition, the natural phytoalexin has been shown to modulate the synthesis of haepatic apolipoprotein and lipids [8], to inhibit platelet aggregation [3,39] and the production of proatherogenic eicosanoid by human platelets and neutrophils [31], and to have chemopreventive potential, consistent with the observation of inhibition of tumor initiation, promotion, and progression [17,18]. Our results confirm that treatment with β 25–35 amyloid peptide induces oxidative stress and causes DNA damage in HUVECs, reinforcing the hypothesis of amyloid cascade as a cause of AD damage involving of ROS production. Treatment with the peptide is able to generate important cellular damage after 24 h of treatment, particularly at higher concentration (20 μg/ml), in agreement with other authors [23]. Aβ-induced damage at endothelial level could contribute to altering repair and regeneration processes, in turn accelerating the progression of deposition in vascular dementia such as in AD. As confirmation of the above, we also observed oxidative damage in the cells treated with serum of AD patients, in particular in the presence of 10% activated serum. Under these conditions we found, in fact, LDH release, a reduction in mitochondrial enzymatic activity, ROS production and an increase in MDA levels and DNA damage. Mitochondrial alterations, in the presence of active redox iron, which is increased in both NFT and Aβ deposits [38], represent a primary cytoplasmic source for ROS generation. Several experimental studies suggest that alterations in oxidative mitochondrial metabolism found in the brain, as in other tissues may play an important role in AD. Acting as a by-product of the respiratory chain, ROS increase promotes damage to mtDNA, proteins and membrane phospholipids with further impairment of respiratory machinery in a sort of catastrophic vicious cycle, promoting cell death. Many experimental data indicate that complex IV is particularly affected by AD in the brain [35]. In addition Al^{+3}, which also accumulates in neurons containing NFT, is known to stimulate iron-induced lipoperoxidation [30]. Our results support the idea that the serum of AD patients contains factors that can interfere with cellular proliferation and with the physiological reactions of HUVECs, probably damaging the repair mechanisms of this endothelial tissue. As hypothesized, the crude extract of black grape skin contrasts the oxidative effects of β 25–35 amyloid peptide and AD serum in HUVECs, evidencing the beneficial effects of this natural compound against the neuro-pathological diseases. In vivo animal studies have demonstrated the protective effects of quercetin [36], resveratrol [41], and (+)catechin [13] in various models of neurotoxicity. In addition, it has been reported in literature that the intraperitoneal injection of resveratrol protects the brain from oxidative damage, indicating that this molecule crosses the hematoencephalic barrier [6].

Conclusion

Our results indicate the effectiveness of combined micronutrients present in black grape skin (vitamin E + vitamin C + resveratrol + quercetin) in initiating, probably, an adaptive transcriptional response which augmenting the ‘‘antioxidant status’’ of the cells, prevents DNA damage. These in vitro studies provide a biochemical basis for a preventive and therapeutic use of the nutritional supplement in neurodegenerative diseases.

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