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Milk whey protein dietary supplementation as tool for modifying oxidative stress in neurological disorders

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TITOLO
Integratori alimentari con proteine da siero di latte come strumento per la modifica dello stress ossidativo nelle malattie neurologiche

KEY WORDS
Milk whey protein dietary supplementation, oxidative stress, neurodegenerative disorders, mitochondrial diseases, myotonic dystrophy, amyotrophic lateral sclerosis

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Integrazione con proteine da siero di latte nella dieta, stress ossidativo, malattie neurodegenerative, malattie mitocondriali, distrofia miotonica, sclerosi laterale amiotrofica

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Summary
Milk whey proteins, produced by a proprietary technique involving microfiltration and low-temperature pasteurization of milk, can be used as an effective oral supplement protecting against oxidative stress in both healthy subjects and in different types of diseases with the purpose to. This result is mainly due to their high capacity to donor cysteine residues which confer them a significant antioxidant activity. The possible fields of application of a milk whey protein dietary supplementation include also several neurological diseases characterized by different etiology but with a common pathogenic mechanism due to altered cellular oxidative status. In this article we reviewed our experience with the milk protein as support treatment in patients affected by some neurometabolic or neurodegenerative disorders, such as mitochondrial diseases, myotonic dystrophy and amyotrophic lateral sclerosis.

Riassunto
Le proteine da siero di latte, prodotte mediante un procedimento che prevede la microfiltrazione e la pastorizzazione a bassa temperatura del latte, possono essere utilizzate come integratori alimentari efficaci sia in soggetti sani sia in diversi tipi di malattie con lo scopo di proteggere dallo stress ossidativo. Tale effetto è dovuto principalmente alla loro elevata capacità di donare residui di cisteina che conferisce una significativa attività antiossidante. Il possibile campo di applicazione della integrazione orale con proteine da siero di latte include anche molte patologie neurologiche caratterizzate da diversa eziologia, ma accomunate da uno stesso meccanismo patogenetico, ovvero da un alterato status ossidativo della cellula. In questo articolo abbiamo descritto la nostra esperienza con integratori orali a base di proteine da siero di latte come trattamento di supporto in pazienti affetti da alcune patologie neurometaboliche o neurodegenerative, come le malattie mitocondriali, la distrofia miotonica e la sclerosi laterale amiotrofica.

Introduction

The potential involvement of free radicals or oxidative damage as consequent of mitochondrial dysfunction in the pathogenesis of human disease has received an enormous amount of study in the last decade (1-4). Free radicals are atoms or molecules with unpaired electrons in their outer orbits, making them highly reactive with macromolecular structures, leading to cellular injury. Free radicals resulting by normal metabolism are balanced by endogenous mechanisms reducing their formation or enhancing their inactivation (5, 6). The imbalance between the production of reactive oxygen species (ROS) and the ability of the endogenous systems to remove them or repair the cellular damage leads to a condition known as oxidative stress (7). This term describes the adverse effect of oxidative reactions induced by free radicals within biological organisms. Cellular ROS derives from the mitochondrial aerobic metabolism (8-10). The mitochondrial respiratory chain transfers electrons from NADH or FADH through a series of electron acceptors to the final oxygen acceptor, with production of energy and water (11), but the electrons loss from the mitochondrial respiratory chain leads to an incomplete reduction of molecular oxygen during oxidative phosphorylation with ROS production (10). Excessive accumulation of ROS can damage biomolecules, including lipids, proteins and nucleic acids leading to a progressive decline in physiological function (12).

In particular, ROS can attack proteins causing their carbonylation, which is an irreversible oxidative damage, often leading to a loss of protein function and protein aggregation (13). Moreover, peroxynitrite modifies protein activity through protein tyrosine residues nitration (11). Free radicals are able to cause lipid peroxidation catching electrons from the lipids, often affecting polyunsaturated fatty acids, in the cell membranes, ensuing in degradation of lipids and cell damage (15). Furthermore, ROS can damage DNA causing mutations resulting in inheritable disease, cancer and aging (16). Such oxidative stress has been implicated in the pathogenesis of various diseases affecting the human nervous system.

Antioxidants are endogenous or exogenous compounds that either reduce the formation of free radicals or react with and neutralize them, thus potentially protecting cells from oxidative injury.

Between endogenous antioxidant molecules it can be count enzymes (for example superoxide dismutase, catalase, glutathione peroxidase), antioxidant compounds (also found in diet: α-tocopherol and ascorbic acid), other antioxidant substances (uric acid, melatonin and glutathione (GSH), the major intracellular antioxidant), antioxidant cofactors (selenium, coenzyme Q_{10}), precursors and derivatives of antioxidant compounds and enzymes (acetylcysteine, polyethylene glycol superoxide dismutase). Metal chelators naturally occurring plant substances (flavonoids in Ginkgo biloba and black tea, lycopene in tomatoes), synthetic free radical compounds and compounds with other primary beneficial therapeutic effects can be considered as exogenous antioxidant with free radical scavenging activity (17). Antioxidants may be lipid soluble (for example vitamin E) or water soluble (for example vitamin C) and possess varying degrees of blood-brain barrier (BBB) penetrance. Antioxidants that readily pass through the BBB are good therapeutic candidates for use in neurologic disorders (17).

Glutathione is one of the major intracellular antioxidant compound and its biosynthesis depends on the intracellular availability of cysteine (18). According to this evidence, it has been described that supplementation with N-acetylcysteine enhanced muscle cysteine and GSH availability and attenuated fatigue during prolonged exercise in endurance-trained individuals (21) but several significant dose depending adverse effects with this treatment

have been described (conjunctival irritation, dysphoria, sleepiness, cough, palmar and facial erythema, dyspepsia and nausea) (20). A milk whey-based oral supplement with a relative abundance of glutamylcysteine has been shown to increase intracellular GSH concentrations because of cysteine enters the cell more readily in the form of glutamylcysteine moiety (18). This supplement is a protein concentrate which consists of several compounds, including albumin, lactoferrin, and a-lactalbumin, which are rich in cysteine (the oxidized form of cysteine) residues and in glutamylcysteine, substrates for endogenous GSH biosynthesis (18).
In this paper, we review our experience with milk whey proteins supplementation as cysteine donor food integrator in some neurometabolic or neurodegenerative disorders, such as mitochondrial diseases, myotonic dystrophy and amyotrophic lateral sclerosis, neurological disorders characterized by evidences of oxidative stress as pathogenic factor in determining cell damage.

Mitochondrial myopathies

Mitochondria are highly dynamic and pleomorphic intracellular organelles composed of a smooth outer membrane surrounding an inner membrane of significantly larger surface area that, in turn, surrounds a protein-rich core, the matrix (21). The majority of mitochondrial polypeptides are encoded in the nuclear genome, synthesized in the cytosol and imported into the mitochondria post-transcriptionally (22) although mitochondria contain their own genome and protein synthesizing machinery (23).
Human mitochondrial DNA (mtDNA) is a circular, double-stranded molecule, which contains 37 genes: 2 rRNA genes, 22 tRNA genes, and 13 structural genes encoding subunits of the mitochondrial respiratory chain (24). The main mitochondria role is the synthesis of ATP generated via glycolysis or by oxidation of glucose to ethanol or lactic acid (25). Electrons from oxidative substrates are transferred to oxygen, via a series of redox reactions, to generate water (26). In this process, protons are pumped from the matrix across the mitochondrial inner membrane through the electron transport chain (ETC), which consists of four multimeric complexes -I to IV- plus two small electron carriers, coenzyme Q -or ubiquinone- and cytochrome c. This process creates an electrochemical proton gradient, which is utilized by complex V (ATP-synthase), which generates ATP flowing back as protons into the matrix (27).
Mitochondria are also involved in many other metabolic processes including the biosynthesis of amino acids, vitamin cofactors, fatty acids, iron-sulphur clusters (28), cell signalling (29) and programmed cell death (30).
Mitochondrial diseases are disorders caused by impairment of the mitochondrial respiratory chain (24) characterized by genetic error either mtDNA or nuclear DNA (nDNA) (31). The extraordinary variability of clinical presentations from pure myopathies to multisystemic disorders with involvement of visual and auditory pathways, heart, gastro-enteric system, central nervous system, skeletal muscle (32) is attributed to the peculiar rules of mitochondrial genetics, especially heteroplasmy and the threshold effect (24). These diseases are not as rare as commonly believed: their estimated prevalence of 10 to 15 cases per 100,000 persons (24). Diagnosis often requires a complex approach with measurements of serum lactate, exercise testing, magnetic resonance spectroscopy, muscle histology and ultrastructure, enzymology and genetic analysis. The pathological hallmarks are the presence of ragged red fibers, ragged blue fibers and cytochrome c oxidase (COX)-negative fibers in muscle tissue (33). The respiratory chain is the main intracellular source of energy but also of ROS

deriving from electrons loss through the mitochondrial respiratory chain with incomplete reduction of molecular oxygen (10).
The proximity to the inner mitochondrial membrane, where oxidants are formed, makes the mtDNA particularly sensitive to oxidative damage; furthermore the mtDNA is not protected by histones; in this way it can be more easily damaged by free radicals resulting in oxidative mtDNA base modifications that can lead to bioenergetic dysfunctions, generating a vicious cycle of mitochondrial dysfunction, increased ROS production, and cellular death (34).
A deficiency of reduced GSH in skeletal muscle from patients with mitochondrial disorders, either as a consequence of diminished ATP availability or of increased oxidative stress has been reported (35). Moreover several studies have showed a decrease in the activities of the ETC complexes following GSH depletion (36, 37). Given those evidences it has been postulate that the decreased GSH concentration may contribute to the progressive nature of mitochondrial disorders (35) and that the replacement of cellular GSH could has beneficial therapeutic effects.
On this base our research group examined in a double-blind crossover study with 30-days supplementation with a whey-based cysteine donor (Prother™ SOD, 10 g/day) in blood samples from 27 mitochondrial patients and 42 controls, the modification of lactate concentration during cycle ergometer aerobic exercise, muscular strength, quality of life and the values of some peripheral oxidative stress markers, in particular advanced oxidation protein products (AOPP), ferric reducing antioxidant power (FRAP) and total GSH (38). AOPP is one of the main markers of *in vivo* proteins oxidation impairment, closely related to the levels of dityrosine, this in turn a hallmark of oxidized protein, and to pentosidine, a marker of enzymatic protein glycation tightly (39). AOPP values were spectrophotometrically determined according to Witko-Sarsat protocol (39). FRAP is a sum of biological antioxidant defined as "any substance that, when present at low concentration compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (40); the estimated relative contributions to the FRAP value of fresh plasma are 15, 5, 10, and 5% for ascorbic acid, α-tocopherol, protein, and bilirubin, respectively. FRAP values were spectrophotometrically assessed according to Benzie and Strain protocol (40). The content of total GSH was determined according to the enzymatic assay described by Tietze (41) and modified by Baker and co-workers (42).
Patients performed series of 3-min of exercise (60-70 revolutions/min). The workload was increased from 25 W until 70% of the predicted normal maximal power output (pnPOmax), or until the highest workload at which cycling could be maintained. Patients also underwent the MRC (43) scale of muscle strength and SF-36 (44) scale of quality of life (38).
We observed, compared to controls, a significant increased of AOPP values while FRAP levels did not differ between the two groups. Resting plasma FRAP and AOPP significantly increased and decreased, respectively, as well as straight after and 15 min after the end of the effort (recovery). Total GSH levels significantly increased both at rest and after exercise (38). Treatment did not modify lactate concentration, clinical scale (MRC) or quality of life (SF-36). Our results reinforce the notions that in mitochondrial diseases oxidative stress is relevant and cysteine donor treatment is able to modify mitochondrial patients' redox profile.

Myotonic Dystrophy

Myotonic dystrophy (DM) is an autosomal dominant multisystemic disease with a severe phenotype characterized by a variable

involvement of skeletal muscle, heart, eyes, brain and endocrine system (45). Two different DM phenotypes are described associated to two different genetic loci. DM type 1 (DM1) is due to an abnormal expansion of CTG triplet in 3' untranslated region of the myotonin protein kinase gene, located on chromosome 19q13.3. The mutation responsible for DM type 2 (DM2) is a CCTG-repeat expansion in intron 1 of the gene coding for the ZNF9 gene on chromosome 3q21.3 (46). Although the pathogenic mechanisms involved in DM1 are still unknown (47), a role of oxidative stress is demonstrated by data indicating increased free radicals production, accumulation of peroxidation products and reduced antioxidant defences (48, 49). Moreover, as Ihara and colleagues reported (48), the serum lipid peroxide concentration was increased in DM1 patients and tended to increase further as the disease progressed. Furthermore evidences came from Usuki and Ishiura (50) suggesting that mild expanded CTG repeats in myotonin protein kinase (MtPK) may amplify cell susceptibility to oxidative stress. Targeted disruption of the MtPK gene in mice in fact has produced only late-onset myopathy with mitochondrial abnormalities (51, 52); another paper of Usuki and colleagues demonstrated that cells transfected with MtPK cDNAs containing a different number of CTG repeats may evoke different apoptosis/proliferation-differentiation signaling pathways after oxidative stress (53). Only few therapeutical trials based on antioxidant principles, such as selenium-vitamin E and coenzyme Q_{10}, have been performed on DM1 (54-56), with controversial results.

Recently (*unpublished data*), we analyzed the oxidative status in 14 DM1 patients during an open label trial before and after one months of treatment with a cysteine donor food integrator (Prother™) (10 g/die). DM1 patients were clinically scored according to the Muscular Disability Rating Scale (MDRS) (57). We observed, compared to 10 matched controls, that before treatment mean blood levels of AOPP and FRAP were significantly higher in patients than in controls while total GSH was not different in the two groups. After 30 days of treatment, AOPP levels significantly decreased while FRAP and GSH levels did not show any change. In DM1 after 30-day treatment, average MDRS score was not significantly different from the basal value. These results showed that in DM1 increased oxidative stress occurs and that an antioxidant cysteine-donor diet supplement might have potential beneficial effects (Figs. 1, 2, 3).

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a neurological disease of unknown origin characterized by a selective degeneration and death of upper and lower motor neurons, initiating in mild adult life and almost invariably progressing to paralysis and death over a 1-5 year time course (58). The clinical manifestations reflect the involvement of both upper and lower motor neurons. ALS diagnosis is based on the El Escorial criteria revised, and mainly based on clinical features in four body regions (59). About 90% of ALS patients are sporadic, whereas 10% are familial. In this group, a mutation in the SOD1 gene (on chromosome 21) that codes for the Cu,Zn-superoxide dismutase (SOD), an enzyme that catalyzes the dismutation of superoxide to molecular oxygen and hydrogen peroxide (60), has been found in a subset of 20% of patients (61). The symptoms and pathology of familiar ALS patients with SOD1 mutations closely resemble those of patients with sporadic ALS suggesting that the mechanisms of neurodegeneration for sporadic ALS and familiar ALS share common components (62). Several potential mechanisms of motor neurons degeneration in sporadic ALS have been proposed. These include the involvement of environmental factors (63), genetic

Figure 1 - AOPP values in DM1 patients before and after treatment

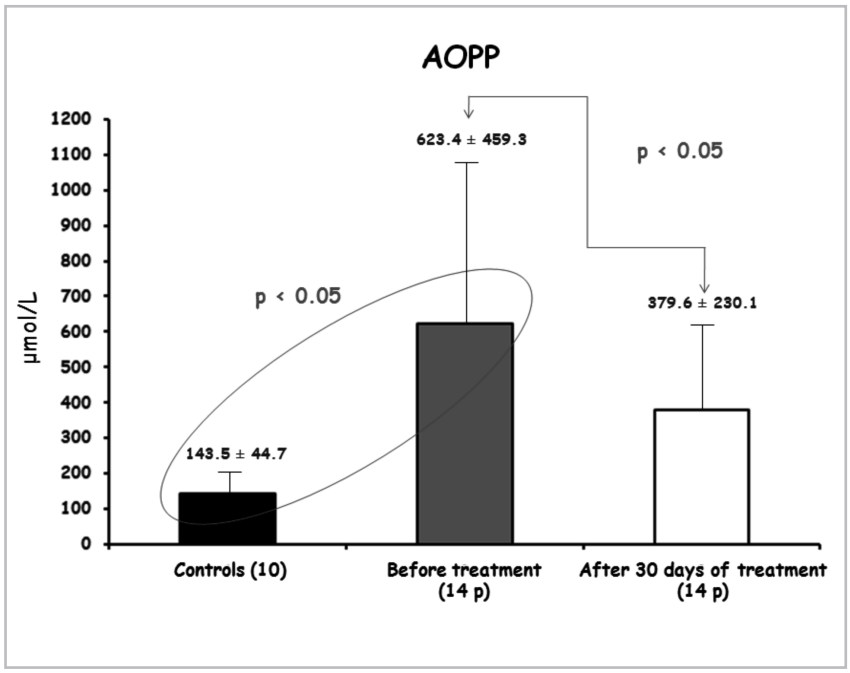
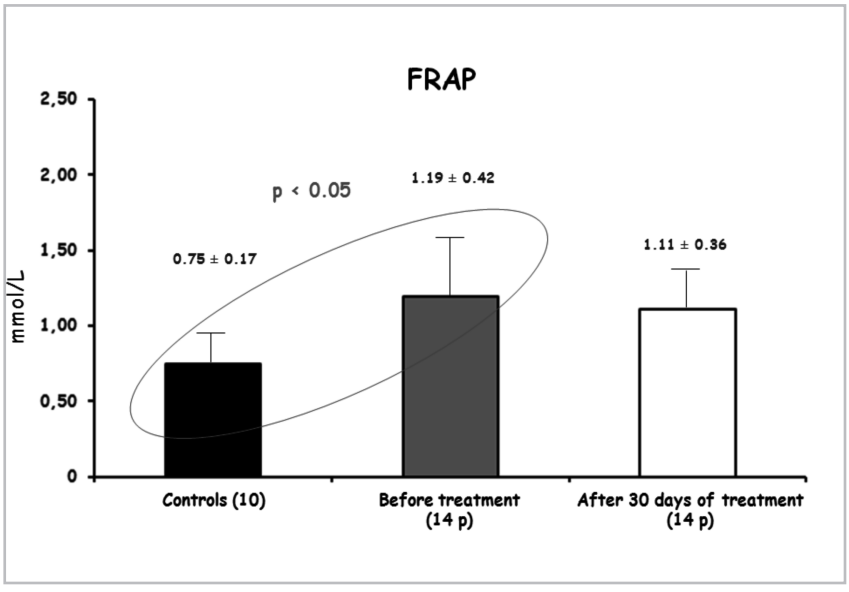


Figure 2 - FRAP values in DM1 patients before and after treatment



factors (64), autoimmune phenomena (65), increased oxidative stress (66), glutamate toxicity (67), viral infections (68), protein aggregation (69), mitochondrial dysfunction (70), cytoskeletal abnormalities (71), impairment of axonal transport (72) and pro-apoptotic alterations (73). Although the pathogenetic mechanisms leading to ALS are still unclear, accumulating evidences indicate that oxidative stress is involved in the pathogenesis of this disease. Increased oxidative stress appears to be an early and sustained event in association with motor neuron death in ALS (74), although the specific mechanism leading to oxidative damage on motor neurons remains to be defined. Moreover, whether oxidative stress is a primary cause of pathogenesis in ALS, or is merely a consequence of the disease, has long been debated.

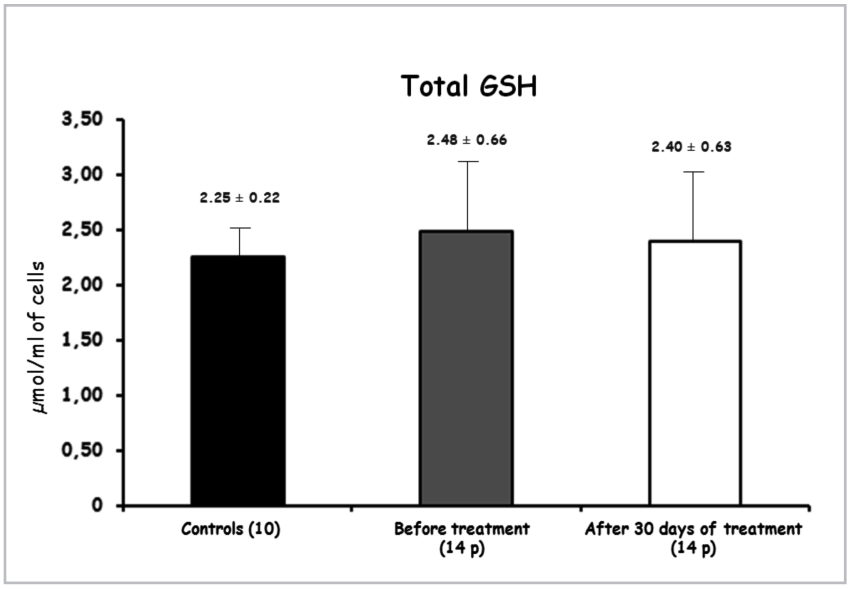
Several studies have showed an increased oxidative damage in spinal cord and motor cortex motorneurons (75) in both sporadic and SOD1 familial ALS post mortem tissue (76-78). Moreover oxidative damage to DNA measured by levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), and lipid oxidation occurs (79, 80). Imbalance of oxidative stress has been reported also in cerebrospinal fluid (CSF) of ALS patients (81, 82). We have also observed (33) a significantly decrease of the total antioxidant

capacity and an increased content of oxidation protein products both in CSF and in plasma in ALS patients compared to controls.

Although the evidence for oxidative stress is strong in ALS, the role of altered GSH metabolism is less clear. There is strong evidence that elevated extracellular levels of glutamate in ALS cause excitotoxicity and contribute to motorneurons death. Alterations of GSH metabolism may enhance generation of ROS and, in the case of increased GSH hydrolysis by γGT, also increase concentrations of glutamate (83). However, GSH concentrations in the cortex or spinal cord of ALS patients are not supported by convincing reports. The increase of GSH-binding sites in the spinal cord of ALS patients (84, 85) may be interpreted as an up-regulation caused by a deficiency of GSH.

On this base, in our Clinic we have performed an open label trial with a food-integrator cysteine donor (Prother™, 10 g/die) in 16 ALS patients before and after 3 months of treatment in order to analyze the modification of some peripheral oxidative stress markers (86). In particular, we assessed blood levels of total GSH, AOPP and FRAP. AOPP and FRAP were also measured, besides resting condition, during an incremental hand-grip dynamometer muscle exercise test, performed in the two experimental conditions. Clinical evaluation was effectuated at the beginning and at the end of the treatment using ALS-Functional Rating Scale-revised (87) and MRC scales.

Figure 3 - Total GSH values in DM1 patients before and after treatment



Patients performed an incremental muscle test with a hand-grip dynamometer for the assessment of AOPP and FRAP in the resting state, at the end of the exercise and after 15 minutes recovery, before and after cysteine donor treatment. Total GSH was assessed only in the resting state before and after the treatment. At the start of each experiment the patient performed 3 brief maximal efforts on the hand grip dynamometer. The highest tension recorded was taken as the maximal voluntary contraction (MVC). Fifteen minutes later the test begun with a first bout at 10% of MVC, then continued trough successive 10% increments, up to 70% of MVC. Each bout consisted of 1 minute intermittent contractions on the hand grip dynamometer followed by a 2 minutes rest.

Patients performed the exercise test at baseline and after 45 days of treatment. After 3 months of treatment only 3 of the enrolled patients were able to performed the hand grip exercise because of the very rapid progression of the disease.

We found that, in resting condition and before therapy, compared to 7 matched controls, FRAP was unchanged, while total GSH was significantly decreased (Fig. 4) and

AOPP was significantly increased (Fig. 5) in ALS patients. After 45 days of treatment, during incremental exercise, ALS patients showed a significant decrease of peak exercise blood AOPP levels, compared to pre treatment condition. After 3 months of treatment also basal blood AOPP levels showed a significant decrease as compared to the basal pre-treatment value in ALS patients (Fig. 5).

However, clinical evaluations after treatment, showed a decrease of ALS-FRS-r and MRC scale scores compared to pre-treatment's one and reductions in MVC due to the very rapid disease progression.

The evidence of a significant total GSH reduction in ALS patients confirms the hypothesis that GSH depletion could be an important factor in the disease pathogenesis. Moreover, our results confirm beneficial effects of antioxidant cysteine-donor therapy to modify blood levels of oxidative stress biochemical markers.

Conclusion

The biochemical imbalance between oxidant and antioxidant compounds has been shown to be involved in physiological aging process as well as in the pathogenesis of many diseases. Among these, neurological disorders characterized by different etiology, clinical presentation and progres-

Figure 4 - Total GSH values at resting condition before and after treatment in ALS patients

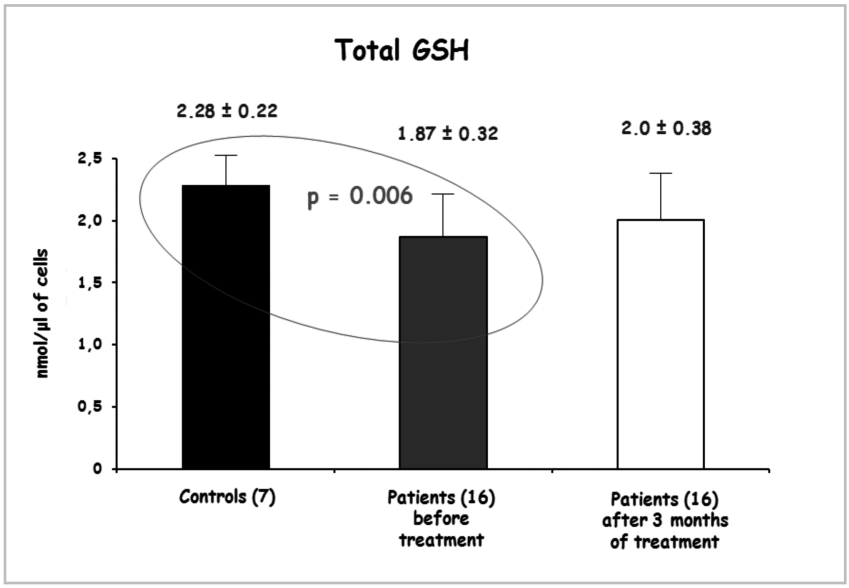
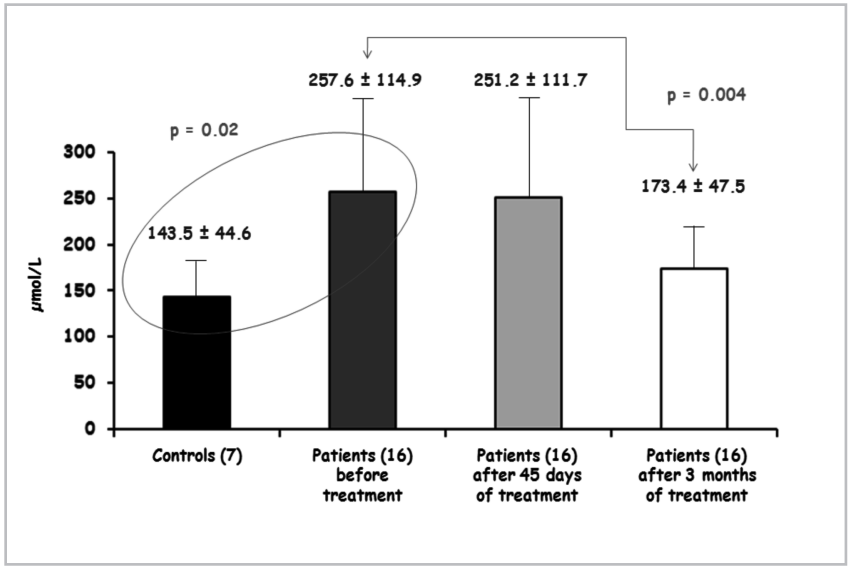


Figure 5 - AOPP values at resting condition before and after treatment in ALS patients.



sion but unified by the common mechanism of oxidative stress related cell damage can be considered. Based on this, several therapeutic attempts using molecules with antioxidant properties have been performed to test clinical and laboratory effects in these neurological disorders. Among them, dietary milk whey based antioxidant supplementation has been assessed in diseases as mitochondrial myopathies, DM and ALS, neurodegenerative or neurometabolic disorders involving both the central nervous system and peripheral nerves and skeletal muscle. Although not definitive, these studies show beneficial effects of the milk whey protein dietary supplementation and reinforce the notions that oxidative stress can play an important role in the pathogenesis of these diseases.

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