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ORIGINAL ARTICLE

Oxidative stress neuroinflammation and cellular stress response in sensorineural hearing loss: novel nutritional therapeutical approaches

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Summary. This study is intended to validate the hypothesis that changes in the redox state of glutathione, the major endogenous antioxidant, associated with the abnormal expression and activity of cytoprotective vitagenes, which in normal conditions are expressed only at low level may represent a critical factor, involved in the physiopathological changes associated to degenerative damage occurring in cochlear diseases. Moreover modulation of stress responsive vitagenes by nutritional antioxidants can be an effective therapeutic strategy to minimize consequences of oxidative stress associated to the pathogenesis and course of sensorineural hearing loss. One therapeutic approach can be antioxidant substances, as cisteina and superoxide dismutase supplementation to burst vitagenes and confer neuroprotection. The damage caused in the inner ear by oxidative stress can induce apoptosis and necrosis of both the hair cells as neurons of the spiral ganglion. Reactive oxygen species (ROS) and free radicals are formed not only as by-products of various metabolic pathways but also for exposure to ototoxic substances such as aminoglycosides and cisplatin, for hypoxia/ischemia and to exposure to noise. Although the mechanism of production of ROS within the cochlea has not yet been precisely identified, it is conceivable that mitochondrial dysfunction and consequent burst in oxidative stress are major causative factors. Consistent with this notion, it is known that the base of the cochlea is more vulnerable to oxidative damage resulted from exposure to ototoxic substances than the apical portions. The difference in survival between the basal outer hair cells and the apical ones appear to be due to a significantly lower level of glutathione in the basal outer hair cells than the apical, a phenomenon that makes it easier basal cells vulnerable to damage from free radicals.

Key words: oxidative stress, neuroprotection; inner ear, free radicals, hearing loss

Introduction

The concept that low levels of harmful substances can induce protective responses is an old one, exemplified by Evelyn Witkin'sground-breaking discovery of the bacterial SOS system.

Mild exposure to environmental stress, as well as mutations that activate stress-response pathways, can also extend the lifespan of many animal species. In some cases, cell-protective pathways are activated by the generation of reactive oxygen species (ROS) within the animal. For example, low levels of the toxin paraquat or inhibition of mitochondrial superoxide dismutase, both of which increase mitochondrial superoxide levels, extend the lifespan of Caenorhabditis elegans.

Lifespan is also extended in response to elevated ROS levels when C. elegans is fed the glucose analog 2-deoxyglucose. Likewise, ROS is part of the mechanism by which TOR inhibition extends lifespan in yeast, and acute inhibition of the daf-2 insulin/insulin-like growth factor 1 (IGF-1) receptor extends life in

C. elegans. ROS also plays an important role in the extension of lifespan caused by a mild inhibition of respiration in worms and flies.

Whether ROS can promote life extension in mammals is not yet clear, but ROS does enhance immune function in mice carrying respiration mutations, and it enhances glucose uptake in response to exercise in humans.

Neuroinflammation, a specialized immune response that occurs in the nervous system, has been connected to chronic neurodegenerative disorders of the central nervous system (CNS) characterized by gradual loss of neurons from specific regions of the CNS. This cell loss is believed to explain the cognitive and motor deficits suffered by patients with neurodegenerative disorders.

Brain inflammation has been linked to many of these diseases, including amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), Parkinson's disease (PD) and, particularly, Alzheimer's disease (AD) (1).

Increasing evidence indicates that factors such as oxidative stress and disturbed protein metabolism and their interaction in a vicious cycle are central to AD pathogenesis (2-4). To adapt to environmental changes and survive different types of injuries, brain cells have evolved networks of responses which detect and control diverse forms of stress. Consistent with this notion, integrated survival responses exist in the brain, which are under control of redox dependent genes, called vitagenes, including heat shock proteins (Hsps), Sirtuins, Thioredoxin and Lipoxin A4, that actively operate in detecting and controlling diverse forms of stress and neuronal injuries (5). LXA4, a metabolic product of arachidonic acid, is considered to be an endogenous "stop signal" for inflammation, and has potent anti-inflammatory properties in many inflammatory disorders, such as nephritis, periodontitis, arthritis, inflammatory bowel disease (6). Chronic inflammation sustains the progression of Alzheimer's disease (AD), however identification of mechanisms capable of resolving the pro-inflammatory environment stimulated by AD pathology remains an area of active investigation (7).

Treatment with the pro-resolving mediator aspirin-triggered lipoxin A4 (ATL), resulted in improved cognition, reduced Aβlevels, and enhanced microglia phagocytic activity in Tg2576 transgenic AD mice (8). Furthermore, LXA4 levels are reduced with age, a pattern significantly more impacted in 3xTg-AD mice (9). Moreover, aspirin-triggered lipoxin A4 upregulation enhanced cognitive performance of 3xTg-AD mice, an effect associated with reduction of Aβand phosphorylated-tau (p-tau) levels, as well as microglial and astrocyte reactivity (6).

Activation of LXA4 signaling could therefore serve as a potential therapeutic target for AD-related inflammation and cognitive dysfunction. LXA4 action is mediated by LXA4 receptor (ALX) on cellular membrane, which is known as formyl-peptide receptorlike 1 (FPRL1) (10). Microglia play an essential role in innate immunity, homeostasis, and neurotropic support in the central nervous system.

In Alzheimer disease (AD), these cells may affect disease progression by modulating the buildup of b-amyloid (Ab) or releasing proinflammatory cytokines and neurotoxic substances. Discovering agents capable of increasing Lipoxin A4 (LXA4) and consequently of increasing Ab uptake by phagocytic cells is of potential therapeutic interest for AD. Lipoxin A4 (LXA4) as an endogenously produced eicosanoid, inhibits neutrophil recruitment and activation, reduces many cell responses evoked by pathogens and pro-inflammatory cytokines, blocks the generations of pro-inflammatory cytokines and toxic compounds including ROS, thereby promoting resolution of inflammation, and acting as an endogenous "braking signal" in the inflammatory process (11).

Glutatione, which have been used in traditional medicine for thousands of years (12, 13), has been reported to possess various biological actions, including antitumor, immunomodulatory, antioxidant effects (14, 15).

A limited number of detailed ultrastructural studies have demonstrated significant reductions in dendritic innervation densities, raising the possibility that neurotoxicity plays an important role in the pathology of sensorineural hearing loss. Increasing evidence suggests that oxidative stress is involved in the development of cochlear hearing loss with cellular damage and apoptotic cells. Furthermore, it is well known that reduced expression and/or activity of antioxidant proteins lead to oxidative stress, accelerated aging and neurodegeneration.

While excess reactive oxygen species (ROS) are toxic, regulated ROS, however, play an important role in cellular signaling.

The ability of a cell to counteract stressful conditions, known as cellular stress response, requires the activation of pro-survival pathways and the production of molecules with anti-oxidant, anti-apoptotic or pro-apoptotic activities. Among the cellular pathways conferring protection against oxidative stress, a key role is played by vitagenes, which include heat shock proteins (Hsps) heme oxygenase-1 and Hsp70, as well as the thioredoxin/thioredoxin reductase system.

Heat shock response contributes to establish a cytoprotective state in a wide variety of human diseases, including inflammation, aging and neurodegenerative disorders.

Given the broad cytoprotective properties of the heat shock response there is now strong interest in discovering and developing pharmacological agents capable of inducing stress responses. When appropriately activated, cellular stress response has the capability to restore cellular homeostasis and rebalance redox equilibrium. Activation of antioxidant pathways is particularly important for neural cells with relatively weak endogenous antioxidant deafeness, such as spiral ganglion neurons which are a centrally involved in the pathogenesis of cochlear sensorineural hearing loss.

Similar problem seems to intervene also in all diseases in which there is a vascular and neurological distress combined, as for example happens in the cerebellar angioreticuloma (16).

Perturbation of redox status, overloading of the product of polyunsaturated fatty acid peroxidation (hydroxynonenals, HNE) or protein oxidation (DPNH) can disrupt redox homeostasis. Moreover it is known that normal auditory function depends on maintenance of the unique ion composition in the endolymph. Hence, carbonic anhydrase in the inner ear has been suggested to play an important role in maintaining the ion concentration and regulating fluids of the inner ear. Basal levels of oxidants are indispensible for redox signaling to produce adaptive cellular responses such as vitagenes linked to cell survival, but at higher levels are detrimental to cells, contributing to aging and to the pathogenesis of numerous age-related diseases.

Aging is a complex systemic process and the major gap in ageing research remains the insufficient knowledge about pathways shifting from normal "healthy" aging to disease-associated pathological aging. The major complication of normal "healthy" aging is in fact the increasing risk for age-related diseases such as cardiovascular diseases, diabetes mellitus and neurodegenerative pathologies that can adversely affect the quality of life in general, with enhanced incidence of co-morbidities and mortality. In this context global "omics" approaches may help to dissect and fully study cellular and molecular mechanisms of aging and age-associated processes. The proteome, being more close to the phenotype than transcriptome and more stable than the metabolome, represents the most promising "omics" field in aging research. In the present study we exploit recent advances in the redox biology of aging and discuss the potential of proteomics approaches as innovative tools for monitoring at the proteome level the extent of protein oxidative insult and related modifications with identification of targeted proteins.

Protein thiols play a key role in redox sensing, and regulation of cellular redox state is a crucial mediator of multiple metabolic, signalling and transcriptional processes. Under optimal conditions long-term health is maintained by protein homeostasis, a highly complex network of molecular interactions that balances protein biosynthesis, folding, translocation, assembly/disassembly, and clearance. Protein quality control is a critical feature of intracellular homeostasis. When conformationally challenged aggregation-prone proteins are expressed, the resulting unfolded or misfolded proteins are rapidly degraded via the ubiquitin–proteasome pathway.

The ability of a cell to counteract stressful conditions is also known as cellular stress response or heat shock response, which is an ancient and highly conserved cytoprotective mechanism. Production of heat shock proteins, including protein chaperones, is essential for the folding and repair of damaged proteins, serving thus to promote cell survival conditions that would otherwise result in apoptosis. There is significant interest in the discovery and development of small molecules that modulate heat shock responses and parallel stress response pathways for therapeutic purposes.

The cellular stress response is regulated at the transcriptional, translational and post-translational

levels. The major regulator of the heat shock response genes is the heat shock transcription factor 1 (HSF1) which is kept in a latent state by an inhibitory complex of stress-proteins, and plays a key regulatory role in response to environmental stress, development, and many pathophysiological conditions, including cancer, ischemia-reperfusion injury, diabetes, and aging (17)

Mammalian cells contain at least 3 HSF family members, HSF1, HSF2 and HSF4. Neurons appear to be deficient in the heat shock response while retaining the ability to express such HSF proteins. Furthermore, HSF1 fails to be activated in motor neurons even when microinjected with plasmids encoding an HSF1 expression vector, suggesting a block to the HSF1 signal transduction pathways in these cells. HSF1 is repressed under non-stress conditions by a complex containing Hsp90 and other proteins. In this inactive state, HSF1 is a monomer that lacks the ability to bind cis -acting heat shock elements (HSE) in the promoters of HSP genes. Protein stress results in conversion of HSF1 from inactive monomer to DNA binding trimer and remodeling of the inhibitory molecular chaperone complex. Activation of HSF1 by heat shock is a multi-step process, involving multiple inducible phosphorylation, dephosphorylation, acetylation and deacetylation steps, the sum of which results in the transcription of HSP genes.

Extracellular signal input during heat shock involves tyrosine phosphorylation upstream of HSF1, involving the receptor tyrosine kinase HER2 and launching downstream signaling cascades through intracellular kinase Akt . Akt regulates HSF1 at least in part through modulating its association with the phosphoserine binding scaffold protein. The major activator of HSF1 is proteotoxic insults, like heat shock. Misfolded proteins displace HSF1 form the inhibitory chaperone complex, HSF1 trimerizes, becomes phosphorylated and is translocated to the nucleus where it is able to bind to the heat shock element of Hsp genes.

Cellular stress response requires the activation of pro-survival pathways as well as production of molecules endowed with anti-oxidant and anti-apoptotic activities, which is under control of protective genes called vitagenes. Generally, molecular chaperones help hundreds of signaling molecules to keep their activation-competent state, and regulate various signaling processes ranging from signaling at the plasma membrane to transcription.

In addition to these specific regulatory roles, recent studies have revealed that chaperones act as genetic buffers stabilizing the phenotypes of various cells and organisms. This may be related to their low affinity for the proteins they interact with, which means that they represent weak links in protein networks. Chaperones may uncouple protein, membrane, organelle and transcriptional networks during stress, which gives the cell additional protection. The same networks are preferentially remodeled in various diseases and aging, which may help us to design novel therapeutic and anti-aging strategies. Among the cellular pathways involved in the so called "programmed cell life" conferring protection against oxidative stress, a key role is played by the products of vitagenes. These include members of the heat shock protein (Hsp) family, such as heme oxygenase-1, Hsp72, sirtuins and thioredoxin/thioredoxinreductase. Heme oxygenase-1 (HO-1), also referred to as Hsp32, degrades heme, which is toxic if produced in excess, into free iron, carbon monoxide and biliverdin (BV); BV is the precursor of bilirubin, a linear tetrapyrrole which has been shown to effectively counteract nitrosative stress due to its ability to interact with NO and RNS.Sirtuins are a group of proteins linked to aging, metabolism and stress tolerance in several organisms.

Mammalian sirtuins are histone deacetylases, requiring NAD^+ as a cofactor to deacetylate substrates ranging from histones to transcriptional regulators. Through this activity, sirtuins are shown to regulate important biological processes, such as apoptosis, cell differentiation, energy transduction and glucose homeostasis. Recent studies have shown that the heat shock response contributes to establishing a cytoprotective state in a wide variety of human diseases, including inflammation, cancer, aging and neurodegenerative disorders. This condition is also found in childhood neurodegenerative disorders, based immunogenic alterations (18, 19).

Given the broad cytoprotective properties of the heat shock response there is now strong interest in discovering and developing pharmacological agents capable of inducing the heat shock response.

The damage caused in the inner ear by oxidative stress can induce apoptosis and necrosis of both the

hair cells as neurons of the spiral ganglion. Reactive oxygen species (ROS) and free radicals are formed not only as by-products of various metabolic pathways but also for exposure to ototoxic substances such as aminoglycosides and cisplatin, for hypoxia/ischemia and to exposure to noise.

Although the mechanism of production of ROS within the cochlea has not yet been precisely identified, it is conceivable that mitochondrial dysfunction and consequent burst in oxidative stress are major causative factors. Consistent with this notion, it is known that the base of the cochlea is more vulnerable to oxidative damage resulted from exposure to ototoxic substances than the apical portions. The difference in survival between the basal outer hair cells and the apical ones appear to be due to a significantly lower level of glutathione in the basal outer hair cells than the apical, a phenomenon that makes it easier basal cells vulnerable to damage from free radicals.

Various protective mechanisms, however, are involved in the control of cytotoxicity promoted by oxidants, such as:

1) enzymes (heme-oxygenase, superoxide dismutase, catalase, glutathione peroxidase, glutathionereductase);
2) antioxidant compounds (tocopherols, carotenes, ascorbic acid, glutathione, polyphenols).

Long standing oxidative insult causes irreversible lesions of the organ of Corti cells, and increasing evidence suggests that cell damage and apoptosis probably contribute to the progression of the disease.

This study is also intended to validate the hypothesis that changes in the redox state of glutathione, the major endogenous antioxidant, associated with the abnormal expression and activity of cytoprotective vitagenes, which in normal conditions are expressed only at low level may represent a critical factor involved in the physiopathological changes associated to degenerative damage occurring in cochlear diseases.

Moreover modulation of stress responsive vitagenes by nutritional antioxidants can be an effective therapeutic strategy to minimize consequences of oxidative stress associated to the pathogenesis and course of sensorineural hearing loss. One therapeutic approach can be antioxidant substances, as cisteina and superoxide dismutase supplementation to burst vitagenes and confer neuroprotection.

Based on the above considerations, our study aims to assess the extent of plasma oxidative stress and lymphocyte in patients with sensorineural hearing loss and cochlear cytoprotective and antioxidant efficacy in these subjects performed by a product with a cisteina and superoxide dismutase (SOD) content.

Materials and methods

The study was carried out on 30 patients, of both sexes, aged between 20 and 60 years, divided into two groups, A and B.

The group A consists of 15 patients suffering from cochlear sensorineural hearing loss that was been subjected to treatment with cisteina and SOD (Prother SOD®, Previfarma Italy), while group B from 15 patients, also suffering from cochlear sensorineural hearing loss, that instead was not been subjected to any treatment.

Constituted exclusion criteria:

- older than 60 years;
- presence of cardiovascular diseases;
- presence of metabolic disorders and / or parts;
- the presence of external ear pathologies and / or medium;
- presence of alterations of state-acoustic nerve;
- prior learning and / or recent treatment with antioxidant drugs or otherwise active in the compartment cochlear.

All patients were undergo the T0 initial phase, after targeted anamnestic investigation, the Profile of Mood States – POMS – (see annex), specific questionnaire aimed at assessing the emotional and degree of psychological stress status in the index, the basis of specific elements, such as: Tension-Anxiety (TA), Depression-Discouragement (D), Anger-Hostility (AH), Vigor-Activity (V), Fatigue (F), Confusion-Loss (C), in relation to the impairment caused in each subject from hearing impairment.

All patients so were been examined to define the qualitative and quantitative characteristics of auditory function, and in particular by:

- examination ENT;
- test tone audiometry;
- speech audiometry;
- impedenzometry examination.

Such instrumental examinations were been aimed at defining not only the extent of hearing, but also the location of the sensorineural hearing loss, to seat cochlear or retrocochlear.

Each patient, either in group A that of group B, will also be subjected to blood sampling for the purposes of biochemical evaluation in the blood (plasma and lymphocytes) of specific markers of:

1) cellular oxidative stress;
2) lipid and protein oxidative metabolism;
3) cellular stress response (vitagenes);
4) glutathione status (reduced glutathione (GSH), oxidized glutathione (GSSG) and GSH/GSSG ratio);
5) lipoxin A4;
6) redox proteomics.

It was then conducted correlative evaluation of the redox status, cellular stress response, lipoxin A4 and redox proteomics and the qualitative and quantitative characteristics of sensorineural hearing loss, in order to identify a possible direct correlation between the extent of oxidative stress and severity of hearing loss involvement .

In patients of group A was wrap the administration of the compound in the T1 phase cisteina + SOD assess the neuroprotective, anti neuroinflammatory potential and thus protection against the cellular degeneration in general and, particularly, in the inner ear.

The product was been dispensed in the measure of 2 sachet/day in 2 daily administrations, for 2 consecutive months.

In step T2, at 2 months of distance from the T1 phase, we have evaluated in patients in Group A and B the degree of:

- cellular oxidative stress, by blood sampling and biochemical evaluation;
- evolutivity trend auditory function.

The correlative analysis was been aimed at highlighting the antioxidant properties of the compound administered and its effects at the cellular level as well as at the clinical level, audiological function. Patients were been undergo blood sampling for biochemical analysis.

Blood was been collected from heart puncture and transferred into tubes containing EDTA as an anticoagulant. Immediately after sampling, 1 ml the blood was been centrifuged at 3000 x g for 10 min at 4°C to separate plasma from red blood cells and 4 mL were utilized for lymphocytes purification.

Lymphocytes were been purified by using the FicollPaque System following the procedure provided by the manufacturer (GE Healthcare, Piscataway, NJ, USA).

Tissue homogenate was been centrifuged at 10,000 g for 10 min and the supernatant used for HO-1, HO-2, Hsp72, Hsp60, TRX1, TRX, Sirt-1, Sirt-2, UCP1, carbonyls and HNE levels determination, after dosage of proteins as described below. Aliquots (30 μg) of protein extract was been separated by SDS-PAGE, transferred to nitrocellulose membranes and then probed with antibodies. Appropriate secondary antibodies were been used and the immunoreactivity visualized using ECL (Amersham Biosciences).

Immunodetection of HO-1, HO-2 and Hsp72 was been performed by using, respectively, a polyclonal rabbit anti-HO-1 (SPA-895) and anti-HO-2 (OSA-200) antibodies (Stressgen, 1:2000 dilution in PBS, pH 7.5) and a monoclonal mouse anti-Hsp70 antibody (SPA-810, Stressgen). When probed for Hsp60 and TRXr proteins, polyclonal goat anti-HSP60 antibody (sc-1052, Santa Cruz; 1:1000 dilution in PBS, pH 7.5) and polyclonal rabbit anti-TRXr-1 antibody (07-613, Upstate) were been used, respectively.

For immunodetection of HNE, membranes were been incubated for 2 h at room temperature with anti-HNE (anti-4-hydroxy-2-Nonenal Michael adducts (393205, Calbiochem, San Diego, CA). Carbonyls groups were been estimated with a rabbit anti-Dinitrophenyl (DNP) antibody (V0401, DAKO; 1:1000 dilution in PBS pH, 7.5). All blots were been then visualized using a horseradish peroxidase-conjugated goat anti-rabbit or anti mouse IgG. A goat polyclonal antibody specific for b-actin was been used as a loading control (sc-1615 product of Santa Cruz; 1:1000 dilution in PBS pH, 7.5). For detection, the membranes were been incubated with a horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (IgG), followed by ECL chemi-luminescence (Amersham).

The amount of inducible HO-1, Hsp72, Hsp60, Trx1, Trx, carbonyls and HNE was been quantified by scanning Western blot imaged films with a laser densitometer (LKB -Ultroscan, XL model). Multiple exposure of each blot was been used to ensure linearity of the film response.

GSH and GSSG were been measured by the NADPH-dependent GSSG reductase method as previously reported (5). Lymphocytes were been homogenized on ice for 10 s in 100 mM potassium phosphate, pH 7.5, with 12 mM disodium EDTA. For total glutathione, an aliquot (0.1 ml) of homogenates or plasma was been immediately added to 0.1 ml of a cold solution containing 10 mMDTNB and 5 mM EDTA in 100 mM potassium phosphate, pH 7.5. The samples were been then mixed by tilting and centrifuged at 12,000 g for 2 min at 4°C. An aliquot (50 μl) of the supernatant was been added to a cuvette containing 0.5 U of GSSG reductase in 100 mM potassium phosphate and 5 mM EDTA, pH 7.5 (buffer 1). After 1 min of equilibration, the reaction was been initiated with 220 nmol of NADPH in buffer 1 for a final reaction volume of 1 ml.

The formation of a GSH-DTNB conjugate was been then measured at 412 nm. The reference cuvette containing equal concentrations of DTNB, NADPH and enzyme, but not sample was been used.

For assay of GSSG, aliquots (0.5 ml) of homogenate or equal volume of plasma was been immediately added to 0.5 ml of a solution containing 10 mM N-ethylmaleimide (NEM) and 5 mM EDTA in 100 mM potassium phosphate, pH 7.5. The sample was been mixed by tilting and centrifuged at 12,000 g for 2 min at 4°C. An aliquot (500 μl) of the supernatant was been passed at one drop/s through a SEP-PAK C18 Column (Waters, Framingham, MA), previously washed with methanol followed by water.

The column was been then washed with 1 ml of buffer 1. Aliquots (865 μl) of the combined eluates were been added to a cuvette with 250 nmol of DTNB and 0.5 U of GSSG reductase. The assay then was proceed as in the measurement of total GSH. GSH and GSSG standards in the ranges between 0 to 10 nmol and 0.010 to 10 nmol, respectively, added to control samples were been used to obtain the relative standard curves, and the results were been expressed in nmol of GSH or GSSG, respectively, per mg protein in the case of lymphocytes or per ml plasma.

The identification of protein bands by 1-D or 2-D gel was been obtained by the construction of the peptidic map essentially using mass spectrometry MALDI-TOF (Matrix -Assisted Laser Desorption Ionization - Time Of Flight). The protein band was excised from the gel, digested in situ with appropriate proteolytic enzymes, and the resulting peptide mixture was been directly analyzed by MALDI-MS

The identification of the proteins was been performed using the mass values accurate of the peptides determined by MALDI-MS. These values, in fact, together with other parameters such as the type of protease used for the hydrolysis or the molecular weight of the protein presumed from the gel of SDS, were been introduced in some research programs available in the network (ProFound , MASCOT, MS -Fit).

The mass values recorded on the spectra were been compared with those from the theoretical digestion of all proteins in the database, allowing the identification of the protein.

Results

The analysis of the results with regard to the examinations of the auditory function highlighted:

Profile of Mood States (POMS): it has been shown in group A subjects, after treatment, an average improvement of subjective parameters related to the psycho-emotional status of the patient. In patients of group B, instead, were not recorded particular changes.

Tonal audiometry: presence in all subjects in T0 initial phase, both in group A that the B group, of sensorineural hearing loss. The tonal interest was centered on medium high frequencies, with an average intensity of 55 dB loss. In all subjects in T1 phase no significant changes have been identified with regard to both the frequency range, is the averageloss in dB.

Speech audiometry: in all subjects in T0 initial phase, both in group A that the B group, threshold of intellection, perception that is 100% of the given words, was assumed to be 75db. In subject of A group patients, who received the mixture of cysteine and superoxide dismutase, was found an average improvement of intellection threshold, ie the ability of verbal discrimination, parallel to the improvement of the profile of mood states found in these patients.

In patients in Group B, however, you are not detected any significant change compared to the figure recorded during T0 phase.

Table 1. lymphocyte and plasma content of total glutathione, reduced (GSH) and oxidized (GSSG) pre- and post-treatment; * P ≤ 0.05.* (da 20)

	Plasma		Lymphocytes	
	t_0	t_1	t_0	t_1
		GRUPPO A		
Total GSH	9.22 ± 5.6	11.61 ± 2.7*	7.5 ± 0.6	9.68 ±0.3*
GSH	9.09 ± 5.6	11.32 ± 2.3*	6.48 ± 0.5	9.25 ± 0.6*
GSSG	0.157 ± 0.02	0.141 ± 0.02	0.105 ± 0.01	0.073 ± 0.03*

Impedenzometry examination: in all subjects in T0 initial phase, both in group A that the B group, the impedenzometric examination revealed an average increase in the threshold of stapedial reflexes and the positivity of the Metz test, indicative of cochlear suffering. In all subjects in T1 phase have not identified significant changes in relation to both the average threshold of stapedial reflex, is the positivity of the Metz test.

As a consequence of oxidative stress in tissues and organs, protein and lipid oxidation occur. In this study, protein oxidation has been evaluated by measuring the amount of protein carbonyls (PC) by Western Blotanalysis., DPNH-reactive PC levels resulted, after therapy, significantly higher (P\0.01) in lymphocytes in patients of B group respect to A group.

Following free radical attack, 4-hydroxynonenal (HNE) are formed from arachidonic acid or other unsaturated fatty acids and bind, by Michael addition, to proteins particularly at cysteine, hystidine, or lysine residues. Under conditions of oxidative stress, lipid oxidation, measured by HNE, can also occur in different tissues, including the brain and peripheral blood stream. Examination of HNE levels in lymphocyte esamples has shown, after therapy, a significant elevation (P\ 0.05) of protein- bound HNE in patients of B group respect to A group.

Consistent with others, who showed that oxidative stress and altered thiol status in degenerating brain diseases correlates with systemic redox imbalance and oxidative stress, as in sensorineural hearing loss. Consequently, the content of total GSH, reduced and oxidized glutathione and the GSH/GSSG ratio in the plasma and in lymphocytes of patients with cochlear sensorineural hearing loss was determined as a measure of the antioxidant status and compared with the levels of control group. We report that plasma and peripheral lymphocytes from patients, after therapy, showed a significantly decreased GSH levels (P\0.05) and corresponding significantly increased GSSG levels (P\0.05) in B group patients. These changes significantly decreased the GSH/GSSG ratio in plasma and lymphocytes compared to A group patients (Tab. 1).

Our lab previously demonstrate dupregulation of protective proteins in cells exposed to oxidative stress. Consistent with these prior findings, in the present study we observed an increase dexpression of cytoprotective proteins Hsp72 and a decreased expression in TRX (P\0.01) in lymphocytes of B group patients. Changes in expression of these two proteins seemed to be consequent to a strong oxidant environment underlying the pathogenesis of this neurodegenerative disorder (Fig. 1).

Moreover, it is known that normal auditory function depends on maintenance of the unique ion com-

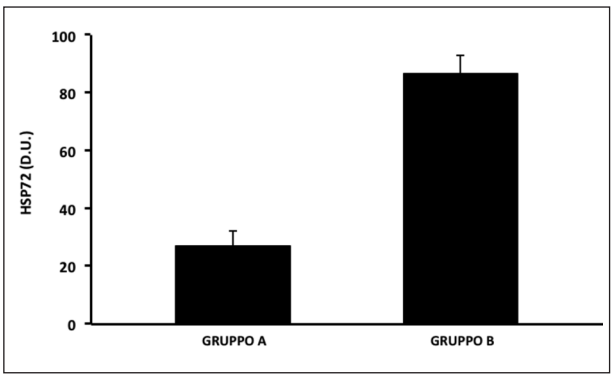

Figure 1. HSP72 expression in the group of subjects A and B in the phase of post-treatment (da 20)

position in the endolymph. Hence, carbonic anhydrase in the inner ear has been suggested to play an important role in maintaining the ion concentration and regulating fluids of the inner ear. Consistent with this notion, we report that in patients of A group a significant controls levels .Results were expressed as means ± S.E.M. OF N=45. Each experiment was performed, unless otherwise specified, in triplicate. Data were analyzed by one-way ANOVA, followed by inspection of all differences by Duncan's new multiple-range test. Differences were considered significant at $P<0.05$.

Discussion

Several causative factors have been considered in the pathogenesis of cochlear dysfunction. The etiological agents, sometimes otherwise interacting with each other, as well as affect the clinical course of the disease, determine interference in the normal homeostatic function of the inner ear neurosensory structures.

Previous studies have shown in cochlear sensorineural hearing loss deterioration amount, as well as morphological structure of the hair cells and ganglion spiral cells.

Additional ultrastructural studies have shown that in the cochlear suffering conditions is detectable in a significant reduction of dendritic innervation density, a factor that increases the likelihood that the activity neurotoxic hosts an important role in the pathogenesis of cochlear sensorineural hearing loss .

The inner ear pathological changes were sometimes also associated with the brain perfusion asymmetry, as well as identified by brain imaging techniques nucleare medicine.

In such a context, growing evidence suggests that the "oxidative stress" might be involved in the pathogenesis of hearing loss and sensorineural cochlear cell damage and cell death by apoptosis are related.

Among the cellular pathways involved in the so-called "programmed cell life" which confer protection against oxidative stress, a key role is played by heat shock protein (Hsp), such as HSP72 and the antioxidant system thioredoxin/thioredoxinreductase.

The data in our possession show that in hypoacusic treated patients, the increase in reduced glutathione group rates, the reduced expression of Hsp 72 and the increased expression of thioredoxin confirm the effect of the treatment as bio-system force capable of counteracting the pro-oxidant cell.

In light of the above remarks it can be said that patients with cochlear sensorineural hearing loss can be in terms of "systemic oxidative stress" and that the induction of protective systems may help restore a biocellular balance, essential for even a check of the evolutionary processes of auditory pathology.

The results of our study have shown that the "oxidative stress" cell, found in the study subjects, suffering from sensorineural hearing loss, can be offset by treatment with a product based on milk serum proteins with high cysteine content, aminoacid, a precursor in the synthesis of glutathione, and superoxide dismutase (Prother SOD®, Previfarma Italy) that possesse an activity of «pro-activation», which follows the induction of bio-cellular mechanisms and these results show the average degree of improvement of verbal intelligibility at speech audiometry and these results seem to be supported by parallel improvement of subjective psicoemotional parameters of related patients.

Found in objectivity agreement, following the objective measurement, using laboratory evaluation of cellular stress, the audiological investigations also showed favorable feedback.. If it is in fact impossible to observe an improvement in tonal audiometric curve, as all patients had sensorineural hearing loss, impossible to recover by administering pharmacological therapy, critical subjectivity against hypoacusic disorders, detected by analysis of a questionnaire which patients have undergone and also the evaluation of speech audiometry, showed a relative improvement of verbal intelligibility and a significant degree of patient satisfaction.

These results would seem to speculate that the reduction in oxidative stress can improve the neuromodulation of cochlear complex and a possible improvement of neural plasticity.

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