The Relationship Between Alanerv® Consumption and Erythrocytes’ Glyoxalases I and II Activities and The Level of Some Serum Markers of Carbonyl Stress in Post-Acute Stroke Patients Undergoing Rehabilitation

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ABSTRACT

Objectives: Diabetes mellitus is one of the most frequent stroke-related comorbid states, and it is characterized by accumulation of reactive carbonyl compounds (RCOs), leading to “carbonyl stress”. This pilot study was aimed to evaluate the effect of the consumption of the nutritional supplement ALA-nerv® on some serum carbonyl stress markers, as well as on the activity of erythrocytes’ glyoxalases in post-acute stroke patients undergoing rehabilitation.

Material and Methods: We created a study population of 28 patients, organized into (-) ALA and (+) ALA groups. Patients from (+) ALA group received ALA-nerv® for two weeks (2 pills/day). All the subjects followed the same rehabilitation program. In both groups, blood samples were taken at the hospitalization and at the discharge moments, respectively. On these samples we assessed lactic acid, fructosamine and RCOs concentrations, as well as the activities of glyoxalases 1 and 2 from erythrocytes’ lysates.

Outcomes: In (-) ALA group the concentrations of fructosamine and RCOs significantly increased (0.90 ± 0.04 vs. 1.02 ± 0.04, p = 0.020; 0.19 ± 0.03 vs. 0.28 ± 0.07, p = 0.027) during the study period. Also, glyoxalase 2 activity decreased in this group (27.04 ± 6.10 vs. 14.43 ± 3.02, p = 0.027). In (+) ALA group, the variation of these parameters did not reach statistical significance. Only, the activity of
INTRODUCTION

The concept of "carbonyl stress" was introduced during the late '90s, by analogy with the well-established concept of oxidative stress (1). It defines a state characterized by accumulation of RCOs like glyoxal (GO), methylglyoxal (MG), glycoaldehyde, hydroxyacylglutathione hydrolase, malondialdehyde (MDA), 4-hydroxynonenal (HNE), acrolein, 3-deoxyglucosone (3DG) etc. These compounds are formed from carbohydrates and/or lipids through oxidative and non-oxidative pathways (2). Thus, glycoaldehyde results from autoxidation of carbohydrates, while MDA and HNE are products of lipid peroxidation. On the other hand, GO and MG arise from both the aforementioned processes. 3DG results from the spontaneous (non-oxidative) decomposition of the fructosamine 3-phosphate, the product of the fructosamine 3-kinase action on fructosamine and other fructosamines from proteins (3).

All these RCOs react with the amino groups of proteins and nucleotides from nucleic acids and through subsequent reactions give rise to a plethora of very complex compounds collectively called AGEs (advanced-glycation end products) and ALEs (advanced-lipoxidation end products) (2,4). The formation of AGEs and ALEs has deleterious effects upon the target biomolecules.

The concept of "carbonyl stress" was introduced in relation to two particular pathological conditions and their complications, uremia and diabetes mellitus, respectively (1,5). The accumulation of RCOs in the aforementioned pathologies is caused by both increased production and impaired detoxification mechanisms.

There are several enzymes involved in the inactivation of RCOs, of which the glyoxalase system is of particular interest (6). It comprises Glo1 (EC 4.4.1.5, 5-D-lactoylglutathione lyase) and Glo2 (EC 3.1.2.6, D-hydroxyacylglutathione hydrolase), that catalyze the GSH - dependent conversion of GO, MG, hydroxyacylglutathione and other α-oxoaldehydes into the corresponding α-hydroxy acids (7). Thus, the product of MG inactivation is D-lactate. This system is present in the cytosol of all mammalian cells, including erythrocytes, and prevents the formation of AGEs and ALEs on proteins and nucleic acids.

It is well documented the link between free radicals and carbonyl stress in different pathological conditions, including diabetes mellitus, uremia, obesity, hypertension and cardiovascular disease etc (8,9). Alteration of the intracellular GSH/GSSG ratio, as a consequence of increased oxidative stress, impairs the in situ activity of the glyoxalase system leading to accumulation of toxic RCOs (10).

OBJECTIVE

The aim of the present study was to evaluate the effect of the nutritional supplement ALAnerv® on the activity of the erythrocytes’ glyoxalase activities in post-acute stroke patients undergoing rehabilitation. Also, it was assessed the concentrations of fructosamine and dicarbonyls as markers of carbonyl stress. The dynamic of these biochemical parameters was followed up for a period of two weeks in two groups of patients, one of which received 2 pills/day of ALAnerv®, while the second one was the control group.

MATERIAL AND METHODS

Design and subjects

For this study we enrolled 28 post-acute stroke patients, which were randomly assigned into (-) ALA (7 females/7 males) and (+) ALA (7
females/7 males) groups. The inclusion criterion used for both groups was the diagnostic of an ischemic or hemorrhagic stroke in the previous 90 days before the enrolment. Cancer, chronic renal failure, chronic inflammatory, autoimmune and haematological disorders, smoking and chronic alcohol consumption were considered as exclusion criteria. Also, patients who were under treatment with vitamins and anti-inflammatory drugs during the two months preceding the beginning of the study and those with a previous cerebrovascular event (cerebral haemorrhage, hemorrhagic infarct, transient ischemic attack) were excluded from the study.

During the study period the subjects from both groups were hospitalized for a standard rehabilitation program. Patients from the (+) ALA group received 2 pills/day of ALAnerv® during this period.

At the beginning of the study the written informed consent was obtained from all patients or from their relatives. This study was conducted in full accordance with established ethical principles (World Medical Association Declaration of Helsinki, version VI, 2002) and it was approved by the ethics review boards of the National Institute of Rehabilitation, Physical Medicine and Balneoclimatology and „Elias” Emergency Hospital, Bucharest (Romania).

**ALAnerv® composition description**

According to the manufacturer specification sheet, one soft gelatine capsule of ALAnerv® contains: α-lipoic acid (300 mg), Borago officinalis (300 mg) which contains 180 mg polyunsaturated fatty acids (linoleic acid and gamma-linolenic acid), D-α-tocopherol on sunflower oil basis (11.177 mg) which contains 7.5 mg vitamin E, thiamine mononitrate 1.259 mg (equivalent of 1.05 mg vitamin B1), riboflavin 1.320 mg (equivalent of 1.2 mg vitamin B2), calcium pantothenate 5.396 mg (equivalent of 4.5 mg vitamin B5), pyridoxine hydrochloride 2.010 mg (equivalent of 1.5 mg vitamin B6), selenomethionine 0.069 mg with 25 μg selenium, fatty acids triglycerides (60 mg), magnesium stearat (14 mg), polyglycerol oleate (10 mg), soya oil and soya lecithine complex (6 mg), food gelatin (177.940 mg), glycerol (82 mg), titanium dioxide (1.520 mg), iron red oxide (0.130 mg).

**Rehabilitation program**

The rehabilitation program consisted of exercise, electrical stimulation, occupational therapy, speech therapy and management of dysphagia if necessary, vocational therapy and counselling. For patients with severe motor deficit initial exposure to orthostatic or gravitational stress (intermittent sitting or standing) and a program based on neural facilitation technique were initiated. Balance (sitting or standing) and transfer training were included in the initial approach. Graded exercise programs were conducted in accordance with the length of the time from the stroke onset and individual functional capacity and the cardiovascular response to exercise. Initial lighter-intensity exercise was prescribed. Increasing the training frequency, duration, or both compensated for the reduced exercise intensity. The program included gait training (body weight-supported treadmill training, treadmill gait training, level ground and step-over obstacle training) as a task-oriented aerobic exercise training to the task of hemiparetic ambulation. For the hemiparetic arm we used a task specific training (reach to grasp) and neural facilitation techniques. Bracing was used for lower limb (ankle foot orthosis) as a compensation for dorsiflexion deficit of ankle, to provide some stabilization for the knee and improve gate pattern. For the upper limb the use of slings and hand orthosis diminished shoulder pain and subluxation and hand spasticity. For the spasticity control we used medication and thermotherapy.

During the hospitalization periods the frequency of training was of 5 days/week. The duration was of 20 to 60 min/day of continuous or accumulated exercise, depending on the patient’s level of fitness. Intermittent training protocols were needed during the initial period of rehabilitation because of the extremely deconditioned level of many stroke patients. The length of the training program was progressive increased to two sessions a day with duration of three hours of accumulated exercise. The goals were: to obtain increase independence in ADLs, increase walking speed and efficiency, improve tolerance for prolonged physical activity, reduce risk of cardiovascular disease, increase ROM of involved extremities, prevent contractures, improve coordination and balance activities.
Electrical stimulation was used to promote muscle activity or to control pain. Management of dysphagia (modified diets, swallowing therapy), speech training and occupational therapy were important steps of our rehabilitation program.

We used the Barthel Index (BI) scale to evaluate the rehabilitation program’s efficiency (11).

Blood samples

We collected blood samples after an overnight fasting of 8-10 hours and serum, plasma and the red-blood-cell pellet were immediately isolated, through centrifugation (5000 rpm, 15 minutes, 4°C). The erythrocytes were washed three times with four volumes of a phosphate buffer – saline solution made by mixing one volume of sodium phosphate buffer (100 mM, pH 7.4) with nine volumes of NaCl (0.9%). The samples were stored in 1.5 mL Eppendorf tubes at -80°C until analysis.

Reagents

All the solvents and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA): glyoxal (GO), methylglyoxal (MG), reduced glutathione (GSH), S-D-lactoylglutathione, Girard’s T reagent, sodium borate, Tris-HCl, Na₂HPO₄, NaH₂PO₄ × H₂O. All the reagents were reagents Ph. Eur. (p.a.).

Biochemical assays

For glucose, lactic acid and fructosamine we used commercially available kits (Spinreact, Girona Spain).

The concentration of RCOs was assessed with a previously described method (12). The results were calculated with a calibration curve made with known concentrations of GO, and were expressed as μmol/L of GO equivalents.

The activities of Glo1 and Glo2 were assessed in erythrocytes’ lysates. These were obtained through the lysis of one volume of red blood cells with four volumes of ice-cold water. The membrane fragments were sedimented through centrifugation (15000 rpm, 20 minutes, 4°C).

The activity of Glo1 was assessed using a previously described method (13). We followed the increase in absorbance at 240 nm due to the formation of S-D-lactoylglutathione from the hemithioacetal of MG with GSH. The hemithioacetal was generated through the incubation of MG (20 mM) and GSH (20 mM) for 10 minutes in an appropriate volume of sodium phosphate buffer (100 mM, pH 6.6) at 37°C. The nominal concentration of the hemithioacetal, which is the substrate of Glo1, was 0.63 mM. The activity was calculated using a molar extinction coefficient of 2.86 mM⁻¹cm⁻¹ and was expressed as units/g of hemoglobin. One unit was defined as the amount of enzyme that catalyzes the formation of 1 μmol of S-D-lactoylglutathione/min under the mentioned assay conditions.

The activity of Glo2 was assessed using the method previously described by Allen et al. (14). We followed the decrease in absorbance at 240 nm due to the hydrolysis of S-D-lactoylglutathione to lactic acid and GSH. The activity was calculated using a molar extinction coefficient of 3.1 mM⁻¹cm⁻¹ and was expressed as units/g of hemoglobin. One unit was defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of S-D-lactoylglutathione/min under the above mentioned assay conditions.

The hemoglobin concentration was evaluated using the method described by Drabkin et al. (15).

All the spectrophotometric determinations were carried on a Shimadzu UV-Vis mini 120 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Statistics

Statistical analysis was carried on using GraphPad InStat 3 software. The results are given as mean ± SEM (standard error of the mean). Nonparametric tests were used because of the low number of samples in each group. Thus, using Wilcoxon and Mann-Whitney tests we compared the means between the two moments of the study and the percentage of variation, respectively. The differences between the two groups in respect to comorbidities and stroke subtypes incidence, as well as medication were evaluated with the Chi-square test. Multiple regression analysis was performed to evaluate the relations between an independent variable (baseline values of the assessed parameters, ALAnerv® treatment, and incidence of diabetes mellitus) and a dependent variable (discharge values of the assessed parameters). In the case of fructosamine, because we used
two different kits from the same producer, in order to avoid inter-assay variability, the statistical analysis was performed using the values obtained after dividing individual values with the mean value of each group. A value of $p < 0.05$ was considered statistically significant.

**OUTCOMES**

For this pilot study we enrolled 28 post-acute stroke patients, which assigned into the previously mentioned (-) ALA and (+) ALA groups. In the (-) ALA group there were 2 patients diagnosed with a hemorrhagic stroke, while in the (+) ALA group there were 3 patients. All the subjects underwent a rehabilitation program for a period of two weeks.

Table 1 summarizes the demographic data, comorbidities and medication of the study participants. The only statistical significant difference between (-) ALA and (+) ALA groups was found in the case of diabetes mellitus incidence.

The dynamic of the assessed biochemical parameters is presented in Table 2. In the (-) ALA group the Glo2 activity significantly decreased (27.04±6.10 vs. 14.43±3.02 U/g Hb, $p = 0.027$), while the concentrations of fructosamine and RCOs significantly increased (0.90±0.04 vs. 1.02±0.04 μmol/L, $p = 0.020$, and 0.19±0.03 vs. 0.28±0.07 μmol/L, $p = 0.027$, respectively) during the study period.

In the (+) ALA group only in the case of lactic acid we found a significant increase during the study period (2.39±0.19 vs. 2.87±0.20 mmol/L, $p = 0.049$).

Fructosamine was the only biochemical parameter for which we found a statistical significant percentage of variation between (-) ALA and (+) ALA groups (14.8%±5.2 vs. –1.0%±13.3, $p = 0.047$).

In the case of the multiple regression analysis we obtained the following models: (1) Glo1 ($R^2 = 0.6947$, $R^2_{adj} = 0.6336$, $F_{3,15} = 11.38$, $p < 0.01$), (2) Glo2 ($R^2 = 0.6729$, $R^2_{adj} = 0.6075$, $F_{3,15} = 10.29$, $p < 0.01$), and (3) RCOs ($R^2 = 0.5520$, $R^2_{adj} = 0.4487$, $F_{3,15} = 5.34$, $p = 0.013$). The results of the regression analysis are presented in Table 3.

As for the rehabilitation efficiency, we found that the BI values significantly increased in both groups (–) ALA, 7.2%±1.2, $p < 0.001$; (+) ALA, 48.4%±17.7, $p < 0.001$), the improvement being more pronounced in the (+) ALA group (7.2%±1.2 vs. 48.4%±17.7, $p = 0.019$) (Table 2). According to the regression analysis, both ALAnerv® treatment and the baseline BI values significantly affected the BI values at the discharge moment.

**DISCUSSION**

The presence of a redox imbalance state in both acute and post-acute phases after stroke is well documented (16,17). On the other hand, diabetes mellitus is one of the major stroke-associated comorbid states, being characterized by increased production of RCOs (9,18).

We designed this pilot study to evaluate the effect of the nutritional supplement ALAnerv® on some plasma carbonyl stress markers in post-acute stroke patients. In order to achieve this, we assessed fructosamine and dicarbonyls concentrations, as well as the activity of erythrocytes’ glyoxalases. These enzymes are involved in the GSH-dependent inactivation of different α-oxoaldehydes to their corresponding α-hydroxyacids. Due to the GSH requirement, the glyoxalase system is very sensitive to changes of the intracellular redox status, reflected in the alteration of the GSH/GSSG ratio.

Despite the fact that ALAnerv® is a complex mixture, it has several compounds with poten-
tial anti-oxidant activity (α-lipoic acid, D-α-tocopherol and selenomethionine as a source of selenium, needed for the function of different selenium-dependent enzymes like glutathione peroxidase).

There is evidence that in vivo α-lipoic acid, after reduction to dihydrolipoic acid, is able to recycle the oxidized forms of some naturally occurring anti-oxidants to their corresponding reduced forms (GSH, α-tocopherol and ascorbic acid) (19). As a consequence, α-lipoic acid could be beneficial for the in vivo correction of the redox status.

We found significant increase of the two carbonyl stress markers (fructosamine and RCOs) in the (-) ALA group during the study period. This could be due to the persistence of the redox imbalance that enhances the formation of RCOs, which at their turn modify plasma proteins leading to the fructosamine formation.

In the (+) ALA group we also found an increasing trend for the concentration of RCOs. Thus, it appears that the administration of the nutritional supplement for only two weeks was not sufficient to counteract the formation of RCOs. In spite of this, the fructosamine concentration had a decreasing trend during the two weeks. Moreover, the fructosamine per-
was positively correlated with the age of the patients.

One pitfall of the present study, a direct consequence of the low number of subjects, is represented by the failure to create and compare two subgroups in the (+) ALA group, patients with diabetes mellitus and patients without this pathological condition. Also, the associations between Glo2 activity and BI values on one hand and the treatment with ALANERV® on the other should be taken with caution due to the low number of patients.

In (+) ALA group it was found an increasing trend for Glo1 activity. Because Glo1 catalyze the rate-limiting step in the glyoxalase pathway, the lack of statistical significance could be a result of the short study period. This, in conjunction with the results of regression analysis suggesting a relationship between Glo2 activity and the ALANERV® treatment suggests that this nutritional supplement could help the correction of the glyoxalase system but at a higher dose and a longer time period.

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REFERENCES