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Clinical and biological changes under treatment with lithium carbonate and valproic acid in sporadic amyotrophic lateral sclerosis

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ABSTRACT

The aim of this study was to evaluate the ability of lithium carbonate and valproate cotreatment to modify the survival rate and functional score of patients with definite sporadic amyotrophic lateral sclerosis (ALS). The clinical response of 18 enrolled patients was compared to the evolution of 31 ALS out-patients, carefully paired by age, gender, evolution rate and time of the disease, who never received treatment with lithium and/or valproate. The ALS functional rating scale, revised version (ALSFRS-R), was applied at baseline, 1 month, and every 4 months until the outcome (death or an adverse event). Biochemical markers, such as Cu/Zn superoxide dismutase and glutathione peroxidase activity, and reduced glutathione were assayed in plasma samples obtained at the baseline visit and after 5 and 9 months of treatment. Our results showed that lithium and valproate cotreatment significantly increased survival ($p = 0.016$), and this treatment also exerted neuroprotection in our patients because all three markers reached levels that were not significantly different from the matched samples of healthy donors. The trial stopped after 21 months, when the sample was reduced to under two-thirds, due to the late adverse events of the treatment. The results call for large randomized clinical trials with the dual association, but at low doses to avoid adverse events.

1. Introduction

Sporadic amyotrophic lateral sclerosis (SALS) is considered to be a multifactorial and fatal neurodegenerative disease. Among the main mechanisms involved in the etiology of ALS, calcium-induced excitotoxicity [5], axon transport impairment [28], neuroinflammation [10], a deficit of neurotrophic factors [32], protein aggregation [18] and oxidative stress [24,29] have been implicated. ALS has also been associated with an overproduction of the superoxide anion and a decreased content of reduced glutathione (GSH; [2,9]).

Therapeutic options against ALS are very limited, and riluzole, which possesses an inhibitory effect on glutamate release and a neuroprotective effect against excitotoxic damage, has been confirmed to extend the survival of ALS patients in the early stages of the disease or to extend the time until a tracheostomy is needed [3,20].

Lithium (Li) administration to SOD1 mutant mice G93A has been reported to increase life span, accompanied by enhanced autophagy and an increased number of mitochondria and Lamina VII motor neurons [13].

Protection against glutamate excitotoxicity in cultured brain neurons was potentiated by a higher inhibition of GSK-3β when lithium and valproic acid (VPA) were used in combination [21]. Moreover, cotreatment with lithium and valproic acid (Li + VPA) was more effective than lithium or VPA alone by enhancing the immunostaining of phosphorylated GSK-$3\beta^{Ser9}$ in brain and lumbar spinal cord sections from SOD1 G93A mutant mice that showed the same synergistic effect *in vivo* [12]. Based on the known safety profile of both drugs, we decided to treat ALS patients with lithium carbonate and valproic acid cotreatment. The oxidative response of patients was also recorded to evaluate the ability of Li + VPA to improve the oxidative status of ALS patients.

2. Materials and methods

2.1. Participants

After a review of 98 files from patients with motor neuron disease attending the Nerve and Muscle Clinic at our Institute during the last 3 years, 67 cases had definite ALS, according to the El Escorial clinical

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and neurophysiological criteria [6]. The protocol, previously approved by the institutional committees, started with a complete clinical revision after a standardized invitation to participate. Twenty-one subjects fulfilled all of the inclusion criteria and gave their informed consent to participate in the study. A control group was composed of the other cases with documented definite ALS, paired by age, gender and deterioration rate and with a follow-up of at least 18 months. Subjects from this group were not available for the trial, unreachable at that moment or deceased before the study started, but they had complete records and were followed up at our institute. We excluded patients with extreme values of time of evolution or deterioration rate and co-morbidities such as diabetes. Finally, we analyzed 18 cases under the experimental treatment and 31 controls.

The El Escorial criteria allowed us to consider the definite ALS diagnosis through the presence of upper and lower motor neuron signs in the bulbar and spinal regions and standardized nerve conduction studies showing axonal motor damage, facilitation of the F wave and electromyography with active denervation in more than 3 regions (studies performed by JB).

Before receiving lithium treatment, patients were examined to exclude cases of dehydration, hypothyroidism, alopecia, or prominent tremor. Various factors such as tobacco consumption, drinking well water and the practice of violent or extreme exercise were also recorded. A stable treatment for at least 3 months before the trial, which was then maintained, was required.

The revision of general laboratory and thyroid analyses allowed us to initiate the experimental treatment with 200 mg of valproic acid t.i.d. plus lithium carbonate. Lithium carbonate was administered in increasing doses from 150 to 600 mg during the first 15 days, and after 1 month, doses were adjusted to achieve a plasma lithium concentration between 0.3 and 0.75 mEq/l. Lithium levels were re-analyzed after 5 and 9 months.

2.2. Primary and secondary outcomes

The primary outcome was the occurrence of death or a severe adverse event that required hospitalization. Clinical examinations and functional state analyses on ALSFRS-R [8] were performed during each visit. We also obtained the functional decline rate [19] at baseline and each year, whereas blood and urine samples for subsequent analyses were collected at the first visit (basal) and after 1, 5, 9, and 17 months.

2.3. GSH concentrations

Reduced glutathione (GSH) plasma concentrations were determined using the method described by Hu [17], with modifications from our group [26] with the use of o-phthalaldehyde (OPA) as a fluorescent reagent. Plasma samples were diluted 1:1 (*v*/*v*) with 10% trichloroacetic acid solution, and the tubes were placed on ice for 10 min. Samples were then centrifuged at 4 °C at 100,000 *g* for 15 min to obtain the supernatant for the assay of GSH levels. Approximately 122 μl of the phosphate-EDTA buffer, pH 8.0, was added to 14 μl of the 100,000 *g* supernatant. Samples were then incubated for 15 min with 14 μl of OPA solution. Fluorescence was assessed in a Biotek FLx800 Microplate Fluorescence reader at 350 nm of excitation and 420 nm of emission, with 90% sensitivity. For blank sample analysis, we substituted plasma samples with deionized water and treated these as regular samples. We used pooled plasma samples obtained from healthy controls as an internal quality control. Calibration curves were built for glutathione, and the concentrations were obtained by interpolation of the standard curve. The results are expressed as μmol GSH/l.

2.4. Glutathione peroxidase (GPx) activity

Plasma glutathione peroxidase (GPx) activity was measured according to a method based on the nonenzymatic oxidation of GSH [11,16]. For this purpose, plasma protein content was obtained by the Lowry method using Folin and Ciocalteu's phenol reagent [22]. Samples containing 500 μg of protein were then incubated at 37 °C in 500 μl of phosphate buffer (containing 5 mM EDTA; pH 7.0) + 500 μl of GSH (2.0 mM) + 250 μl of NaN3 (0.01 M) to reach a final volume of 3 ml. After 5 min, 1 ml of H_2O_2 (1.25 mM) was added to the incubation medium, and 3 min later, 1 ml of the mixture was removed and added to 4 ml of metaphosphoric acid. Samples were then centrifuged at 1500 *g* for 30 min. Supernatants (1 ml) were mixed with 1 ml phosphate buffer (pH 7.0) containing 0.5 ml of 5,5-dithiobis-2-nitrobenzoic acid (DTNB). Two minutes after the addition of DTNB, the optical density was determined at 412 nm in a Lambda 20 Perkin-Elmer UV/VIS spectrophotometer. Calibration curves were built using increasing concentrations of GSH + EDTA (pH 7.0) + DTNB, and the concentrations were obtained by interpolation from the standard curve. The results are expressed as μmol GSH/500 μg protein/30 min.

2.5. Superoxide dismutase 1 activity (SOD1)

Plasma SOD1 activity was measured by the xanthine/xanthine oxidase method, as previously described [4]. Plasma samples were diluted in a buffer consisting of 20 mM sodium bicarbonate and 0.02% Triton X-100 (pH 10.2) and centrifuged at 4000 *g* for 10 min; supernatants were collected. Subsequently, 50 μl of the clarified supernatant was added to 950 μl of the reaction mixture, which consisted of 10 μM sodium azide, 100 μM xanthine, 10 μM reduced cytochrome c, and 1 mM EDTA in 20 mM sodium bicarbonate, 0.02% Triton X-100 (pH 10.2). The assay was initiated by adding xanthine oxidase and monitored by measuring the change in absorbance at 560 nm in a Lambda-20 UV/Visible spectrophotometer (Perkin Elmer, Waltham, MA, USA). Sample analysis was performed in duplicate, and the participation of each SOD (SOD1 or SOD2) type was calculated as the total activity minus the activity inhibited by the addition of 5 mM sodium cyanide, given that cyanide selectively inhibits the SOD1 isoform. The results are expressed as international units of SOD1 per milligram of protein (I.U. SOD1/mg protein).

2.6. Cadmium assessment in blood and urine

First morning urine was used for the assessment of urinary cadmium. All of the materials used for sample collection, preparation, and storage were acid washed with 10% HNO_3 and tested for possible cadmium contamination. Cadmium in urine and blood was collected and determined by Graphite Furnace Atomic Absorption Spectrophotometry (GFAAS; Perkin Elmer AAnalyst 600, Shelton CT, USA), according to previous reports by our group [14]. To correct cadmium concentrations in urine samples for the variation in dilution, each sample was adjusted for its creatinine content [30].

2.7. Control samples for biochemical analysis

Plasma samples from patients were analyzed together with those samples provided by age- and gender-matched healthy donors ($n = 7$).

2.8. Statistical analysis

Prior to the comparison between groups, all variables were analyzed for their distribution, using SPSS version 17 software. Bivariate comparisons were performed using Student's *t*-test or the Mann–Whitney *U* test, according to the distribution of continuous variables, and the chi-squared test for categorical variables. Comparative analyses were performed after controlling for age, age at onset and follow-up time. A survival analysis was carried out with the log-rank test to compare treated and untreated patients.