NeuroEPO and cognitive function in Parkinson’s disease in a randomized placebo-controlled and post-trial studies

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Abstract

Background. Cognitive impairment is a feature of Parkinson’s Disease (PD) from the early stages but currently, no treatment for cognitive deficits in PD is available. Erythropoietin (EPO) has been studied for its potential neuroprotective properties in neurologic disorders with a beneficial action on cognition.

Objective: We want to know if NeuroEPO, a new formulation of EPO with low content of sialic acid has effects on cognitive function in PD in a double-blind randomized placebo and after a post-trial intervention.

Methods: The sample was composed of 26 PD patients (HY stages I-II), where 15 received intranasal NeuroEPO for 5 weeks and another age and gender-matched 11 patients were randomly assigned to the placebo. During a post-trial all the sample received 9 months of intensive NeuroEPO treatment. Cognitive functions were assessed using a comprehensive neuropsychological battery before, one week and 6 months after the first intervention and 9 months after the post-trial. The effects of NeuroEPO were evaluated using a multivariate linear mixed-effects model using a latent variable for cognition instead of the raw neuropsychological scores.

Results: We found a significant and direct effect of the dose of NeuroEPO (p=0.00001) on cognitive performance with a strong positive influence of educational level (p=0.0004) and negative impact of age (p=0.007).

Conclusions: This preliminary results showed a positive effect of NeuroEPO on cognition in PD patients.
Introduction

Parkinson’s disease (PD) is a neurodegenerative disorder that mainly affects the motor system, but with a range of non-motor symptoms including cognitive dysfunction, depression, and anxiety. The neural substrates and the pathophysiology of cognitive impairment in PD are not fully known because of their complex nature and the multiple neurotransmitter systems implicated. In the early stages, executive dysfunction and other forms of mild cognitive impairment are features of PD, while in the later stages of PD a significant number of patients develop dementia. Cognitive impairment highly impacts quality of life and depression in PD.

The main risk factor for cognitive deficits is the progression and the severity of the motor symptoms, with age, age of onset, and akinetic-rigid PD subtype as other important risk factors. Currently, the motor symptoms of PD can be well controlled initially by dopaminergic medication and in the later stages with deep brain stimulation, but there is no treatment for cognitive deficits in PD.

Erythropoietin (EPO) is a cytokine, which is known as an important hematopoietic growth factor in tissue oxygenation. It is a glycoprotein hormone that has 165 amino acids, weighs 30.4 kDa, and is a member of the cytokine superfamilies. At present, it is widely used in the treatment of anemia related to premature births, renal failure, cancer, chronic inflammatory diseases, and HIV infections. EPO is believed to have functions that keep tissue oxygenation at adequate levels and is an important hematopoietic factor. Some evidence also established that EPO has other functions such as neuroprotection, although the mechanisms of this are not fully clarified. Preclinical studies in PD and other neurological and psychiatric disorders have suggested the neuroprotection capacity of EPO.

But the treatment of neurological diseases with EPO involves a higher dose and prolonged application, producing adverse effects because of the increment of hematocrit and blood viscosity. For that reason, here we tested the effect of NeuroEPO, a new formulation of EPO with low content of sialic acid (molecule is between 4 and 7 mmol/mL of protein). This molecule is similar to that produced in the brain of mammals but does not have an inducer effect in the synthesis of erythrocytes, maintaining its neuroprotective properties.

NeuroEPO is safe and tolerated in healthy people and PD patients but its neuroprotective effects have been mainly tested in animal models, in-vitro models, and partially reported on the cognitive performance of PD patients. But this is the first attempt to study long-term effects of cognitive effects of NeuroEPO in PD. We consider the importance of neuroprotection against the cognitive decline since 80% of PD patients in late-stage progress to dementia and even in early stages more than 25% have cognitive deficits.

Our study aimed to assess the effect of NeuroEPO on cognitive function in PD patients in two trials, the 6-months randomized placebo-controlled safety trial where the participants received a small dose and later the long-term effect of the drug after a 9 months follow-up post trial using higher dose of NeuroEPO for all the participants.

To test the effect of NeuroEPO we employed the doses of the product received across the two trials such a continuous process could be evaluated using a latent variables longitudinal linear dynamic model.

Methods
The study was part of a randomized double-blind physician-led placebo-controlled trial to evaluate the safety of NeuroEPO in Parkinson’s Disease patients (https://clinicaltrials.gov/ number NCT04110678) with motor and cognitive secondary outcomes measures. It was developed in collaboration between three institutions: International Center for Neurological Restoration (CIREN) and the Center for Molecular Immunology (CIM), La Habana Cuba, and The Clinical Hospital of Chengdu Brain Sciences Institute, UESTC Chengdu, China.

The sample was composed of twenty-six patients (10 women) with a clinical diagnosis of idiopathic Parkinson’s disease according to the UK Brain Bank Criteria. The average age was 53.88 years (SD=7.66) and the duration of disease was 5.5 years (SD=3.49). The patients were in stages I-II of Hoehn and Yahr 21. The inclusion criteria were similar to those employed by our group in this safety trial 14 and to test rHuEPO in another PD patients group 22.

NeuroEPO [CIMAB S.A, Havana, Cuba] was a stabilized liquid formulation, in a unique dose bulb, containing 1 mg (1 mg/mL) of non-hematopoietic rHu-EPO, produced in Chinese hamster ovary (CHO) cells. Each bulb also contains buffer salts, polysorbate 80, sodium EDTA, NaCl, HPMC F4M, and water for injection to complete 1 mL.

The NeuroEPO group included fifteen patients (7 women) who received a weekly 1mL dose of intranasal NeuroEPO for 5 weeks and the placebo group included eleven patients (3 women) who received a similar formulation containing the same ingredients except for EPO. Patients were randomly allocated to NeuroEPO or placebo and the two groups were matched for age and gender.

After the safety trial finished and following the therapeutic obligation principle (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/) inherent to double-blind placebo-controlled trials, the sponsors and researchers conducted a post-trial where they gave access to NeuroEPO to all the patients of the safety trial. This information was disclosed to participants during the informed consent process.

All the patients (n=26) received for 9 months an intensive NeuroEPO treatment of 1mL bulb, 3 times per week, for one month (induction phase) and later 1/2 bulb three times per week, for eight months. This summarized a total of 60ml in all the period in comparison with the 5 ml of the safety trial. Even if all the patients taken part in the post-trial only a subsample of 18 patients (10 from the original NeuroEPO group and 8 from Placebo) attended the final session neuropsychological assessment 9 months after the post-trial intervention. See Table 1 for demographic and clinical data for the samples of both trials.

The dose for each participant is the cumulative amount of drug taken in the time of evaluation: 0 at the baseline for all the patients, 5 mL for NeuroEPO group, and 0mL for the Placebo group in the timepoint 1 (first week) and timepoint 2 (six months) assessments after first intervention respectively. During the post-trial all the patients received in total 60 mg, cumulative 60 mg for the original placebo group and 65 for the NeuroEPO. See Figure 1 for the detailed description of the trial.
|                                | Group A NeuroEPO | Group B Placebo | Total     | Post-trial |
|--------------------------------|------------------|-----------------|-----------|------------|
| **Total**                      | N=15             | N=11            | N=26      | N=18       |
| **Age [mean, SD]**             | 56.4 ± 7.8       | 61.09 ± 6.6     | 58.4 ±7.6 | 57±11.9    |
| **Sex**                        |                  |                 |           |            |
| Male                           | 7 [46.6 %]       | 8 [72.7%]       | 15 [55%]  | 12[66.6%]  |
| Female                         | 8 [53.4%]        | 3 [27.2%]       | 11[45%]   | 6[33.3%]   |
| **Stage Hoehn &Yahr**          |                  |                 |           |            |
| I                              | 4 [26.6%]        | 1 [9.09%]       | 5 [19.2%] | 3[16.7%]   |
| II                             | 11 [73.4%]       | 10 [90.9%]      | 21 [80.8%]| 15[83.3%]  |
| **Duration of illness (years)**| 5.4 ± 3.2        | 5.8 ± 4.06      | 5.6 ± 3.5 | 5.1±4.2    |
| **PD Familial antecedents**    |                  |                 |           |            |
| Yes                            | 6 [40%]          | 3 [27.2%]       | 9 [34.6%] | 6[33.3%]   |
| No                             | 9 [60%]          | 8 [72.8%]       | 17[65.4%] | 12[66.6%]  |
| **Levodopa equivalent Dose LED**| 935.83 ± 302.54  | 939.3 ± 194.0   |           |            |
Figure 1. Time of evaluation in weeks (dark blue). The intervention in grey, and evaluation time points (T1, T2, T3 and T4) in light blue.

This study was approved by the ethics committee of two institutions: International Center for Neurological Restoration (CIREN) and the Center for Molecular Immunology (CIM), Cuba, following all the guidelines for clinical trials of the Ministry of Public Health of the Republic of Cuba. All the patients and caregivers received a detailed explanation about the nature and consequences of the study and signed the required informed consent.

Neuropsychological assessment

Cognitive performance was assessed with a comprehensive neuropsychological battery including global cognitive screening: the Mini-Mental State Examination and the Dementia Rating Scale; memory: the Rey Auditory Verbal Learning test (episodic verbal memory), the subtest letter-number sequencing of the Working Memory Index of WAIS III (Spanish version The Manual Moderno https://www.worldcat.org/title/wais-iii-escala-weschler-de-inteligencia-paradulitos-iii/oclc/54053545) and the Rey Complex Figure, copy and delayed recall/reproduction of the copy (visuospatial ability and non-verbal memory); executive function: Delis-Kaplan verbal fluency, Trail-Making the Stroop color word Interference test and the Frontal Assessment Battery. Each test was administered at the baseline, one week and six months after the first trial and after the post-trial intervention. The tests were scored according to standard procedures. The patients remained on their usual anti-parkinsonism medication (levodopa and dopaminergic agonists) during the neuropsychological assessments.

Motor assessment

To assess the motor symptoms the Unified Parkinson Disease Rating Scale (UPDRS) motor section was employed. The scale was applied in two conditions: “off” and “on” medication.
The analysis of the total score of the UPDRS scale was evaluated within and between groups at all the time-points of the trial using t-tests.

**Statistical guidelines**

**Linear Mixed Effect Model.**

To know the effects of the drug (dose) on cognitive performance, we employed a multivariate linear mixed-effects model in a longitudinal time-dependent repeated data approach. The dependent variable of interest Y (cognitive performance) is assessed repeatedly in the three time-points, which could be the predictor of the effectiveness of the drug.

**Latent variables.**

This model uses a latent variable instead of the discrete neuropsychological scores. In psychological research, the use of implicit variables is common, that is, several measures are collected simultaneously to describe the same phenomenon of interest. For example, a series of psychological tests that assess memory, language, attention, and so on, are often used to assess a person's cognitive level. What is of interest is not the analysis of specific tests individually, but the analysis of the underlying concept that a set of observable markers measure. In this case, the cognitive status. These models containing continuous latent variables are often referred to as "structural equation models". The term structural equation refers to the part of the model that describes the structure of latent phenomena. The script of the multivariate latent process mixed model and the output results are available at [https://doi.org/10.17605/OSF.IO/M8SJP](https://doi.org/10.17605/OSF.IO/M8SJP)

For the statistical analysis, we selected from the comprehensive neuropsychological battery a subset of tests with relative independence and not correlated; given that the variance estimator is biased when the data are correlated. For this reason, we did not select tests and their subtests; and also reduced the number of parameters to 6 for a better fit, according to sample size. We included representative measures in each cognitive domain. Global screening (MMSE and DRS total), Memory (RAVLT (Recognition), Figure Rey recall (Memory), Working memory (letter-number sequencing), and executive function: (FAB total).

The neuropsychological measurements were collected 4 times (T1 baseline, T2 one week, T3 six months after safety trial) for the n=26 patients and T4 one week after the post-trial for 18 patients. For that reason, some variables have missing values, but this is not a problem when using lcemm package as missing values are systematically removed.

**Covariates.**

Initially, we included several covariates such as gender, age, education, PD duration (in years), and stage of PD according to the Hoehn & Yahr scale. All the covariates were tested in the model.

The fixed effect were:
- education: in grades (primary 6, secondary 9, high school 12, university 17)

- initage: the initial age in years when the patient was recruited. This variable was normalized using the subtraction of the initial age (low age in the sample 44 years old)/10.

- dose: the cumulative dose of NeuroEPO received by the patients in each evaluation. 0 at the baseline for all the sample, 5mL after the first trial for the NeuroEPO group, and 0 for the Placebo group. After the post-trial 60mL for the Placebo group and 65mL for NeuroEPO group.

For this model, we did not consider the main effect “group” because in the post-trial all the patients received the drug without distinction. We used the doses, which were evaluated in cumulative points where the quantities were 0, 0, 0, 60 for placebo and 0, 5, 5, 65 for NeuroEPO group.

This binary condition make difficult the continuity effect. For that reason we include the logarithm of the Dose \((\log(Dose + 1))\) to study its effects.

The random effects was:

- subject (their identification number) to control the variability of the repeated measures.

We assume that these psychometric tests are measures with an error of the same underlying latent process, that is the cognitive performance. Change over time of this cognitive performance is described according to the dose received in each moment.

To analyze the longitudinal dataset, we employed the package `lcmm` in R described by Proust-Lima, (2015)\(^{33}\) [https://cran.r-project.org/web/packages/lcmm/index.html](https://cran.r-project.org/web/packages/lcmm/index.html), specifically the function `multlcmm` to fit latent class mixed models for multivariate data with a common underlying process, in this case, the cognitive performance (observations from the neuropsychological assessment).

We tested the model with one latent variable (“ng” in the model) to know which model explains better the variability of the markers (neuropsychological tests).

The Wald test statistic was used to test the fixed effects and the goodness-of-fit we employed the maximum log-likelihood, the Akaike Information Criteria (AIC), and the Bayesian Information Criteria (BIC). The models with the minimum AIC and BIC provides the best fit.

**Results**

**Cognitive performance:**

The first step in this analysis was to design a general latent class mixed model fitted by maximum likelihood with one latent class to explain the variability of the dataset.

See below the formula in R notation:

```
mod <- multlcmm( DRS + FAB + MMSE + sequency + Memory + Recognition ~ log(Dose+1) + education+initage+progression +severity+sex, random =~ 1, subject = 'ID', ng = 1, data = tabla)
```
The Maximum Likelihood Estimates are included in table 2 below.

| Table 2. Fixed effects in the longitudinal model A | Coef. | Se   | Wald | p-value |
|--------------------------------------------------|-------|------|------|---------|
| intercept (not estimated)                         | 0.00  |      |      |         |
| Log(Dose+1)                                       | 0.33  | 0.07 | 4.36 | 0.00001 |
| Education                                         | 0.17  | 0.06 | 2.78 | 0.004   |
| Age at start of trial                             | -0.85 | 0.32 | -2.65| 0.007   |
| Progression                                       | -0.04 | 0.08 | -0.49| 0.62    |
| Severity                                          | 0.67  | 0.73 | 0.92 | 0.35    |
| Sex                                               | 0.34  | 0.43 | 0.77 | 0.43    |

This Model had a good fit (AIC: **2185.5** BIC: **2215.7**).

The model showed a significant linear relation of the latent variable (neuropsychological performance) with the NeuroEPO dose (p=0.00001) in the model represented by the logarithm based 10. Education showed a direct and statistically significant main effect (p=0.004) on the latent variable. This indicates that cognitive improvement was related to higher education levels and higher doses. By contrast, the significant negative effect of age of the patient at the beginning of the trial indicates better results for younger patients (p=0.007). Other covariates included in the model such as sex, the disease progression in years and the PD severity indicated by the Hoehn &Yahr scale didn’t affect the latent variable. To evaluate the relative importance of the markers in the latent process we obtained the percentage of variance explained by the common latent process and the link function, which expresses the individual contribution to the likelihood of a latent process mixed model. See the link functions in Table 3 and Figure 2 and percentage of variance in Table 4.

| Table 3: Link function | Marker                             | coefficient | Se   | Wald  | p-value |
|-------------------------|-----------------------------------|-------------|------|-------|---------|
| Dementia Rating Scale   | 138.9                             | 1.61        | 86.02| 0.00000 |
| Frontal Assessment Battery | 14.03                     | 1.26        | 11.2 | 0.00000 |
| Mini Mental State Examination | 27.29                  | 0.71        | 38.15| 0.00000 |
| WAIS-III Letter-Number Sequencing | 3.77       | 2.19        | 1.71 | 0.086   |
| Visuo-spatial Memory (Rey Figure - recall) | 8.9         | 5.01        | 1.77 | 0.076   |
| Verbal Memory Recognition (RAVLT) | 10.67    | 1.9         | 8.93 | 0.00000 |

**Table 2: Estimated link functions for the latent variable.** The link function of the latent variable with the markers was very significant (p<0.00000 with the exception of WAIS_III sequencing and Visuo-spatial memory Rey Figure.

| Table 4: % Variance explained by the Latent variable for each marker |
|---------------------------------------------------------------|
| Dementia Rating Scale (DRS)                                   | 51.53 |
| WAIS-IV Letter-Number Sequencing                             | 51.38 |
| Frontal Assessment Battery (FAB)                             | 23.98 |
Visuo-spatial Memory (Rey Figure - recall) 16.1
Mini Mental State Examination (MMSE) 13.2
Verbal Memory Recognition (RAVLT) 16.34

Table 3: % Variance explained by each marker for the Latent variable. Here the latent process is explained predominantly by the DRS variance, in second place the letter-numbers sequencing subtest of the Working memory index of WAIS, and the FAB in the third place. The MMSE, the Rey Figure recall, and the recognition subtest of the Rey Auditory Verbal Learning Test (RAVLT) reached around 13 to 16 each.

Figure 2. The General Latent Class Mixed Model. Here the latent variable represented by lambda Λ was estimated for all the subjects (i) and all the timepoints (j). Y is the markers included to estimated the latent variable. The markers are: DRS=Dementia Rating Scale, FAB=Frontal Assessment Battery, MMSE=Mini Mental State Examination, Sequence=WAIS-IV number-letter sequencing, Memory=visuospatial memory recall of Rey figure, Recognition=recognition on Rey Auditory verbal Learning Test. The variance explained for each marker included between lines of contact. The X are the fixed effects: Dose of NeuroEPO, the initial age of the patients, the educational level, the severity of the disease, the disease progression and the sex.

Motor performance:
The motor evaluation using the UPRDS total score during the off and on condition between the baseline and time-point 2, 3 and 4 is subject of another publication in progress. We didn’t include here because the difference between the total score of the UPDRS motor scale at the baseline in comparison with the rest of the time-points between the two groups was not significant.

Discussion
In this randomized placebo-controlled study we examined whether there were any changes in cognitive function in a group of (n=15) PD patients treated with a small dose of intranasal NeuroEPO compared to a placebo group (n=11). Later we did a follow-up after 9 months of NeuroEPO treatment when all of them received an intensive dose of the drug. We found that this formulation of NeuroEPO had a beneficial effect on cognitive performance in all the time-points evaluated using a longitudinal model.
We selected a linear mixed effect model instead of a t-test or ANOVA to test the cognitive performance between time-points instead of evaluating each trial separately. Essentially, because a high number of comparisons invalidate any univariate tests (p fishing) and the small size of the sample; we took advantage of the benefits of using a linear mixed effect model to achieve a more robust analysis.

We preferred to use latent variables to explore changes in the main domains of cognitive performance instead of using raw neuropsychological scores. The original neuropsychological battery was composed of several subtests which were highly correlated, with different metrics to assess the performance. The multiple directions of the neuropsychological tests assessing cognition and the measurement error associated to the observed variables make more difficult to interpret the analysis using individual scores. The solution was to look for a common factor underlying the cognitive performance and select a representative cluster of independent tests sufficient to explain them.

Another reason to use the latent variables is to reduce the ceiling-floor effect of global screening tools such as the MMSE and the DRS, which were included, first because MMSE is the most well-known psychometric test used to describe cognitive aging and the DRS because it has been recommended by the Movement Disorder Society to study cognition in PD. Our preliminary report found\textsuperscript{18} improvement on different tests (FAB, DRS, Rey figure copy and recall) in both groups (placebo and NeuroEPO) when comparing baseline and the two time-points separately. In our opinion, this could be explained partially because the first time-point only six weeks after the baseline, was influenced by practice and learning effects. And second, because of the placebo effect. The powerful placebo effect in PD which is mediated through activation of the dopamine system\textsuperscript{35} is well-recognized and can be elicited by several factors such as expectation of benefits, described in clinical trials of Parkinson's disease\textsuperscript{36}.

The inclusion in the model of the 4 timepoints across the two trials in the longitudinal study in a latent mixed model allow to balance all these factors.

Our results highlighted the positive and direct significance of the doses of NeuroEPO (p=0.00001). Note that the doses employed in the double-blind first trial were modest because the nature of the safety trial obliged us to use small not therapeutic doses. This is a disadvantage, that may have influenced the expression of the cognitive improvement with NeuroEPO relative to placebo. However the inclusion of the post-trial with higher and therapeutic doses compensate this factor. Our results highlighted the protective effect of covariate “education”, and its influence on the NeuroEPO doses, indicating that patients with high educational levels have a better cognitive prognosis. The effect of the age at entry to the trial was significant in a negative direction, showing that the younger the patients were when they started this treatment, the better cognitive outcomes obtained. The progression of symptoms in PD and the associated cognitive impairment are heavily influenced by many factors, including age, in that sense this result is relevant for the clinicians in the recruitment of patients for new protocols. Other covariates such as the sex, years of progression of the disease and severity didn’t showed statistical significance in this trial, but this could be influenced for the imbalance of the factors.

To our knowledge, this is the first study about the cognitive improvement linked to NeuroEPO treatment in PD patients. About the underlying mechanisms which provoke this improvement, we can only mention experimental models. Garzon (2018) confirmed in vitro neuroEPO’s protective effect against neuronal damage induced by excitotoxicity, improving antioxidant activity in the neuron, and protecting it from oxidative stress\textsuperscript{17} and showed how NeuroEPO protects cortical neurons from glutamate-induced apoptosis.\textsuperscript{37} In an APPSwe transgenic mice
model of Alzheimer’s disease, using a low dose of NeuroEPO, cognitive improvement was observed in behavioural outcomes (spontaneous alternation, place learning in the water-maze, and novel object recognition). A post-mortem analysis of the hippocampus or cortex of the animals showed a decrement in synaptic markers of oxidative stress, neuroinflammation, and trophic factors and beta-amyloid load.16

Limitations
The main limitations are the small sample size and the small doses of NeuroEPO, which are both typical characteristics of safety trials. However, to date no treatments have been approved as neuroprotective agents in Parkinson’s Disease. Therefore, our findings, even if preliminary are clinically relevant, given the importance and impact of cognitive deficits on quality of life in PD5. Another limitation is that this study did not include neuroimaging techniques, only behavioral performance. Adoption of a multimodal approach in future studies to shed light on the neural mechanisms explaining this cognitive improvement would be of value.

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Author Contributions
IPI and MGL conducted the clinical trial, recruited the PD patients, and did all the clinical and motor assessments. ECF was in charge of the neuropsychological assessment. DAG, TRO, IST, and LPR designed the NeuroEPO intervention, doses usage, and provided the drug. LS curate the data and did the statistical analysis with AMR, YRL, FAR, MLB, and PVS. MLB, MJ and PVS wrote the final version of the manuscript. All authors reviewed the manuscript.

Competing interests
The author(s) declare no competing interests.

Availability of data and material
The neuropsychological assessment with clinical and demographic variables are included in the dataset NeuroEPOtablePD.xls and the script with the output of the program are available in the OSF repository https://doi.org/10.17605/OSF.IO/M8SJP

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