Forelimb stepping test software development for nigrostriatal dopamine system functionality assessment and motor parameter evaluation.

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***Abstract*— Parkinson disease (PD) characteristic motor dysfunctions are tremors, stiffness, bradykinesia and gait disorders.**
We use an experimental adult male rat model of hemiparkinsonism and investigate chronic estrogen administration effects in the functionality of the nigrostriatal dopaminergic system.
Forelimb akinesia in rats is analogous to limb akinesia and gait problems seen in patients with PD. We have developed a software to test adjusting steps, stepping time, step length and initiation time. The implementation tracks each animal forelimb movement and measures those parameters. It performs statistical analysis and presents data in bar charts or Excel sheets.
The software was validated by correlating manual and automated adjusting steps.
Estrogen treated animals exhibit a higher compensatory mechanism to motor dysfunction, improving the number of adjusting steps and showing less bradykinesia. Also, there is a beneficial effect of estrogen related to a higher average energy that could be enhancing stepping time and forelimb movement magnitude.
In conclusion, this software development and implementation is a worthy tool to *in vivo* infer on forelimb akinesia. By applying it, we obtain a new sensitive approach in the effect of estrogen as a neuroprotective drug in PD as it restores balance, mobility, speed and also diminishes bradykinesia.
***Keywords*— Parkinson Disease, Akinesia, 6 hydroxydopamine, animal model, video monitoring, tracking.**

***Resumen*— Las disfunciones motoras características de la enfermedad de Parkinson (EP) son temblores, rigidez, bradicinesia y trastornos de la marcha. Utilizamos un modelo experimental de hemiparkinsonismo en ratas macho adultas e investigamos los efectos de la administración crónica de estrógeno en la funcionalidad del sistema dopaminérgico nigroestriatal. La acinesia en las patas delanteras de las ratas es análoga a la acinesia de las extremidades y problemas de marcha observados en pacientes con EP. Hemos desarrollado un software para medir los pasos de los animales, el tiempo de paso, la longitud del paso y el tiempo de inicio. La implementación hace un seguimiento de cada movimiento animal de la extremidad anterior y mide esos parámetros. Realiza análisis estadísticos y presenta datos en gráficos de barras o hojas de Excel.**
El software se validó correlacionando los pasos por conteo manual y automático. Los animales tratados con estrógeno exhiben un mecanismo compensatorio más alto ante la disfunción motora, mejorando el número de pasos y mostrando menos bradicinesia. Además, hay un efecto beneficioso del estrógeno relacionado con una energía promedio más alta que podría mejorar el tiempo de avance y la magnitud del movimiento de las extremidades anteriores. En conclusión, este desarrollo e implementación de software es una valiosa herramienta para textit in vivo inferir sobre la acinesia de la extremidad anterior. Al aplicarlo, obtenemos un nuevo enfoque sensible en el efecto del estrógeno como fármaco neuroprotector en la EP, ya que restaura el equilibrio, la movilidad, la velocidad y también disminuye la bradicinesia.
***Palabras clave*— Enfermedad de Parkinson, Akinesia, 6 hidroxidopamine, modelo animal, monitoreo por video , rastreo de trayectoria.**

I. Introduction

Parkinson Disease (PD) is relatively common and serious impairment of health in developing countries all around the world, which affects 1% of the population over 55 years, with a mean onset of 60 years [1]. The disease is degenerative and progressive, and it is expected to become a serious public health problem [2]. The brains of patients suffering from PD show a profound deficit in dopamine levels, because of the loss of neurons in the nigrostriatal pathway. The continuous neuronal degeneration in substantia nigra pars compacta and the dopaminergic deficit in corpus

striatum, generate functional modifications on the basal ganglia system with the consequent characteristic motor dysfunctions such as tremors, stiffness, bradykinesia and gait disorders [3].

It is known that neurosteroids have functional beneficial properties in the Central Nervous System (CNS). Some of them are protection against deleterious agents, enhancement of cognitive abilities and neurogenesis promotion in human and animal models of neurodegenerative diseases. It has also been described that these molecules bind to specific sites of the cell membrane in regions of the CNS; other than those involved in reproductive functions [4]. PD affects men more than women and there are evidences supporting female sexual steroids' neuroprotective properties. Furthermore several neuroprotective and neuroregulatory effects of estrogen have been described for PD [5] [6].

Considering that several reports inform unilateral 6-OHDA lesioned rats as a suitable animal model for behavioral and biochemical evaluation of PD [7] [8], we use an experimental male rat model of hemiparkinsonism induced by 6-OHDA in which we investigate the effects of estrogen in the functionality of the nigrostriatal dopaminergic system.

Particularly, for this work, we have developed a bioinformatic software for forelimb akinesia, in which we assess different parameters (adjusting steps, stepping time, step length and initiation time) analogous to limb akinesia and gait problems seen in patients with PD [9]. By using this computerized technique we'll be able to early, with high sensitivity and in vivo infer about motor parameters related to forelimb akinesia. The software implementation will contribute on describing nigrostriatal dopamine dysfunction and revealing estrogen neuroprotective effects in the animal model of PD.

II. Materials and Methods

A. Animals

We used male Sprague Dawley rats from our breeding colony. They were 60 days old at the beginning of the study, and their weight was 280-340 g. Experimental subjects were housed under controlled conditions of temperature (22 $\pm$ 3 °C) and lighting (12 hour cycle beginning at 7:00 a.m.), with food and water made available *ad libitum*. Animals were kept and handled according to the Guide for the Care and Use of Laboratory Animals of the National Research Council (National Academies, U.S.A, 8th edition, 2011). All efforts were made to minimize animal suffering.

B. Surgical procedures

On postnatal day 60, forty animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) and placed into a stereotaxic frame (David Kopf, USA). The neurotoxin, 6-OHDA was dissolved at a concentration of 2 μg/μl saline in 0.1% ascorbic acid. In order to achieve unilateral lesions of the nigrostriatal system, 20 rats received 6-OHDA injections and other 20 got vehicle (V) into the left corpus striatum. The lesion was performed by injecting the neurotoxic or vehicle with a Hamilton syringe at the following coordinates: AP: +1.2 mm; ML: +2.5 mm; DV: -5.0 mm; TB at 0 mm. The injection was conducted at a rate of 0.5 μl/min and the needle was left in place for another 5 min before it was slowly drawn back. After surgery, when animals were fully recovered, they were taken to a room where they rested for seven days. From PND 67 to 77, 20 rats received chronic treatment with 17β-Estradiol (E= 0.1 μg/kg/day s.c) and 20 got oil (O) as vehicle. Groups were conformed as HP+E (6-OHDA lesion, E treatment, n=10); HP (6-OHDA lesion, O treatment, n=10); E (V lesion, E treatment, n=10); C (V lesion, O treatment, n=10).

C. Forelimb akinesia test

The procedure was similar to the one described by Olsson [9] with some modifications. The test was performed on a smooth-surfaced wooden table with a length of 0.9 m (Fig. 1). Behavioral records were all made during daytime by an observer blinded to the animal condition.

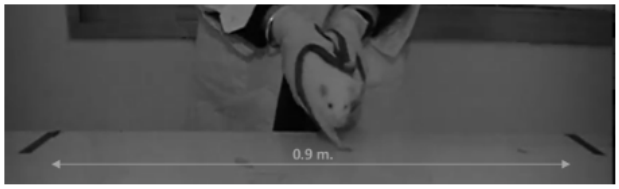

Fig. 1. Animal handling and experimental grip length.

Procedures followed the next outline (Fig. 2); for three days (PND 114,115 and 116), rats were handled by an experimenter and trained for the testing sequence described below. These scores were not recorded. On PND 117, one hour before the test all animals were taken to the behavioral testing room to get used to the environment. The test was video recorded for subsequent software analysis.

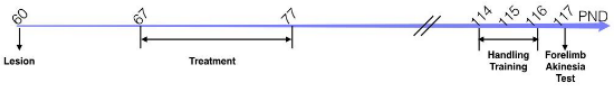

Fig. 2. Experimental timeline.

- Testing sequence.
  As it is shown in Fig. 1, the rat was held by the experimenter with one hand fixing the hindlimb and slightly raising the hind part above the surface, the other hand fixed the forelimb not to be monitored. Then it was moved slowly sideways (0.9 m for 5s) by the experimenter. The testing sequence was right paw forehand and backhand adjusting steps (RP-F, RP-B), followed by left paw forehand and backhand directions (LP-F, LP-B).
  The following parameters were counted for both paws in both directions and the sequence was repeated three times.
  -Initial time [s]: measured until the rat made the first step.
  -Stepping time [s]: measured from first step until the rat reaches the end of the trajectory.
  -The number of adjusting steps was counted for both paws in the backhand and forehand directions of movement.
  -Step length [px]: tested as the magnitude between

each adjusting step.

- Statistical analysis.
  For software validation Pearson correlation coefficient between manual and automated adjusting steps was calculated.
  Statistical evaluation of estrogen effects on forelimb akinesia test depends on the population's nature. For comparisons between the four groups, if data distribution is parametric, results are expressed as means ± SD; data was analyzed with Anova One-Way followed by Tukey's Honest Significant Difference Test. If data distribution follows a non-parametric distribution, results are expressed as medians ± SD and shown as medians. In this case, Kruskal Wallis test followed by Dunn post hoc test was applied.
  Statistical significance level was set at $p< 0.05$.

D. Software implementation

The first stage of this procedure (Fig. 3) consists in conditioning the behavioral testing room according to set parameters. It is an obligation to configure light and temperature according to standard conditions to avoid animals stress. Also, camera speed and resolution must be determined at the highest level available in [frames/s] and pixels, respectively. For this purpose, camera is set in 29 frames/s and 720x480 resolution.

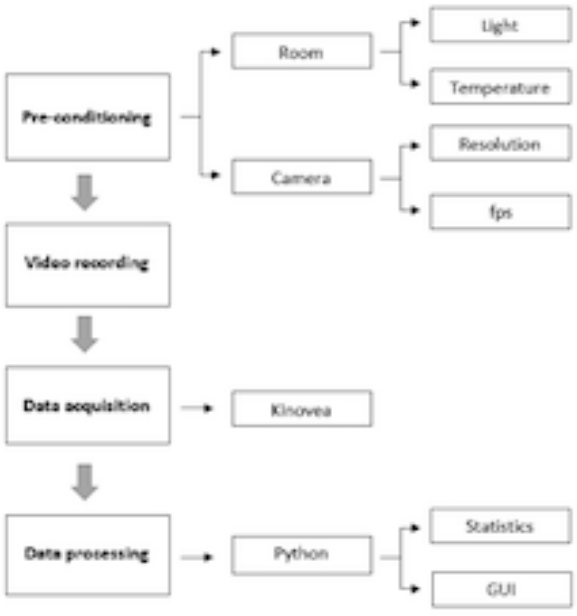

Fig. 3. Software implementation process.

After this pre-conditioning, animal's sequence of testing is performed and video recorded. To track each animal forelimb movement we use Kinovea 0.8.15 [10], a biomechanical analysis video software. These results ase exported from the software as a MS-XML file.

For data processing we developed a software in language Python 2.7.9 [11]. This program consists in measuring stepping test parameters, making statistical analysis and presenting results. To begin, it requires the location of the files exported from Kinovea (this data has to be converted into excel files, XLSX). Each testing group is organized as a file that contains all the animals trajectories. This files must be ordered progressively (Fig. 4). The software asks the user to indicate the number of groups and the amount of animals per group. The user can label each group according to the experimental condition. After this, forelimb akinesia parameters are automatically calculated. This implementation has a module to perform statistical analysis which embodies a set of assumptions concerning on the population distribution and the number of groups tested; and graphic user interface that presents data in barcharts or Excel sheets.

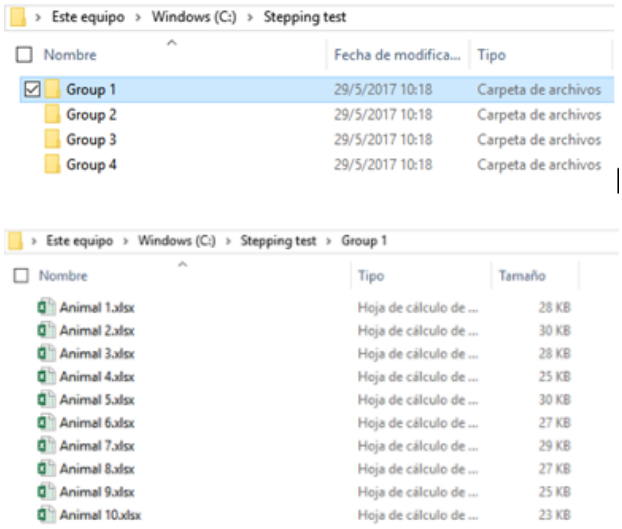

Fig. 4. Kinovea's output. Each file represents the different groups tested (above), inside of it there is an excel sheet per animal analyzed as it is shown below.

III. Results

In order to validate the software we use adjusting steps, as it is the only one parameter that could be compared between manual and automatic measurements. Fig. 5 displays the presence of correlation in values corresponding to this parameter of the testing sequence for all groups, from direct observations and automated software.

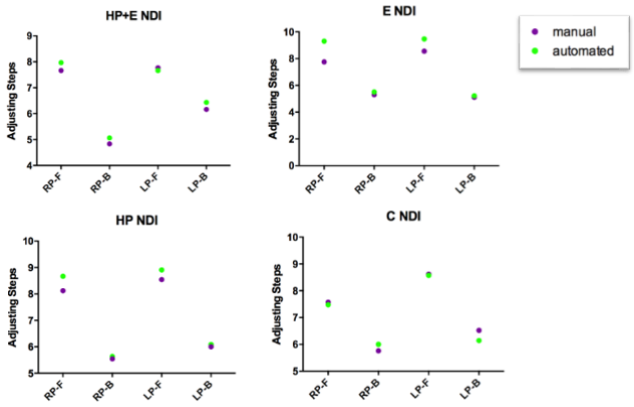

Fig. 5. Results of manual vs automated correlations for each group tested.

As it is shown in Table I, there is a high correlation

between manual and automated values. For all groups, Pearson's coefficient was almost near 1.00 with p-value<0.01. This result represents a strong positive linear relationship among both calculations.

TABLE I
PEARSON'S CORRELATION COEFFICIENTS FOR ALL GROUPS.

Group	Correlation coefficients	p-value	Level of significance
HP+E	0.992	0.008	**
HP	0.998	0.002	**
E	0.979	0.008	**
C	0.987	0.013	**

A. *Estrogen effects on forelimb akinesia test*

- Adjusting steps

  Fig. 6 shows a considerably large difference between C and HP+E with HP and E groups according to the sequence of testing for adjusting steps performance.

  We could see that HP animals displayed a forelimb imbalance pattern and the most affected movement in the sequence of testing was RP-F, the value of this parameter was (8.1±1.09).

  For HP+E group, the number of adjusting steps in the direction RP-F (7.6±1.0) was significantly different ($p-$value<0.001) to RP-B (4.8±1.55) and also to LP-B (6.1±1.92).

  E group also exhibited unequal movements with a similar pattern to HP. In this case, LP-F value (8.67±1.18) was the biggest value in the testing sequence.

  Finally, RP-B value (5.4±1.83) in C group presented was significantly different from RP-F (7.9±1.75) and LP-F (8.85±2.04).

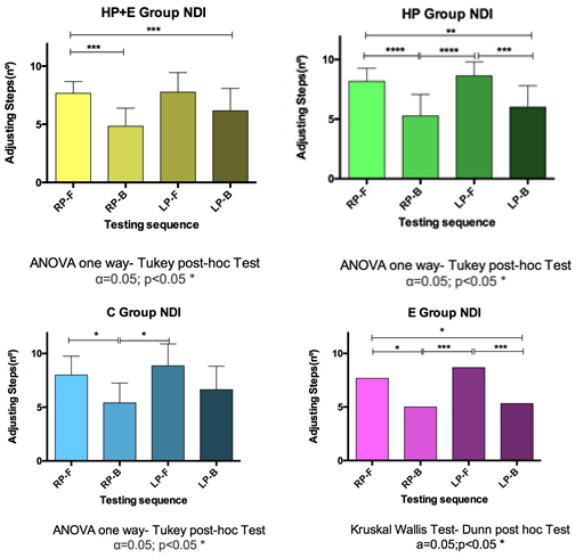

Fig. 6. Adjusting steps according to the testing sequence for all groups tested.

- Initial time

  The duration until the rat made the first step was more than three times larger for lesioned animals. RP-F initial time in HP group (0.36±2.78 s) was three times greater than that same parameter for HP+E (0.2±0.39 s), C (0.22±0.33 s) and E (0.20±0.16 s) group where this parameter never exceeded 1s.

  What is more HP group presented differences among the testing sequence; the highest value was for RP-F, followed by RP-B (0.24±1.94 s), then by LP-B (0.22±1.85 s) and finally LP-F (0.19±2.34 s).

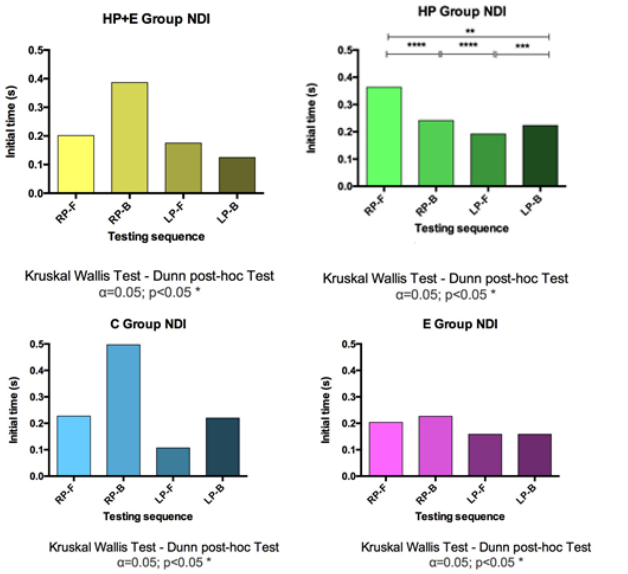

Fig. 7. Initial time corresponding to the testing sequence for all groups tested.

- Stepping time

  The only group that presented significantly differences was HP. In its testing sequence, RP-F (2.59±2.20 s) forelimb movement is the highest, followed by LP-F (2.55±1.70 s). For both paws in backhand direction this parameter was considerably less, (2.28±1.30 s) for LP-B and (2.17±1.41 s) for RP-B respectively.

  The evaluation of this value in the other three groups yields no difference in any testing sequence.

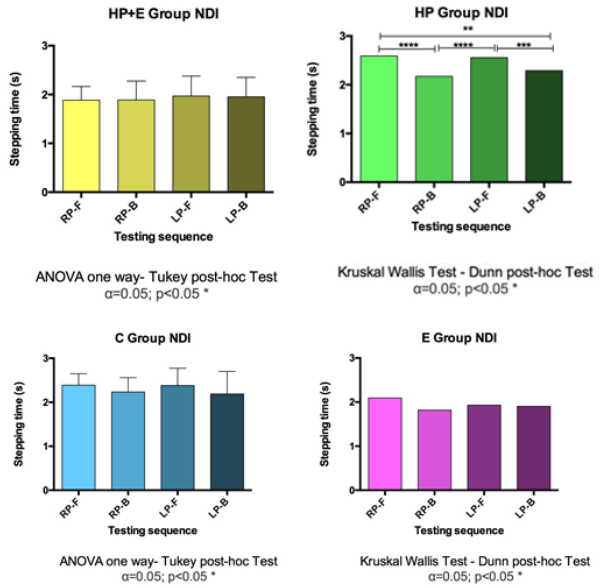

Fig. 8. Stepping time parameter related to the testing sequence for all groups tested.

- Step length

There is no difference (in any group or testing sequence) of the magnitude between each adjusting step. As it is shown in Fig. 9 there is a tendency for RP-B to be the largest value in HP (41.66±57.26 px), C (41.05±40.22 px) and E group (63.61±26.02 px); although this imbalance is not seen in HP+E group. The equivalence to interpret this results in centimeters is: 1px=0.23 cm.

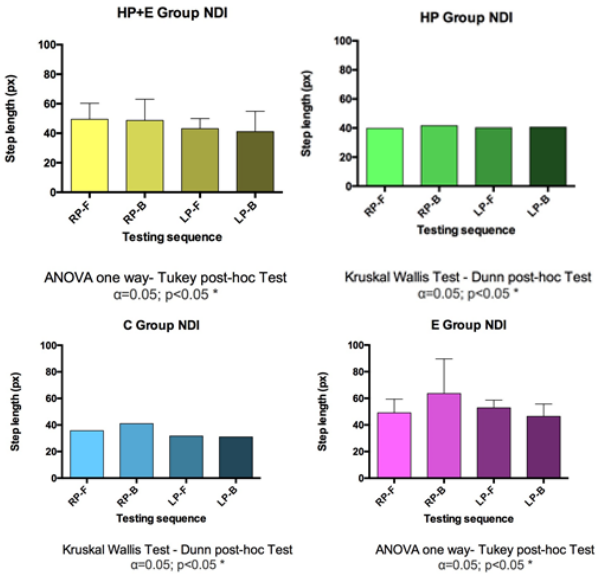

Fig. 9. Step length according to the testing sequence for all groups tested.

IV. Conclusion

There are less differences between the number of adjusting steps in HP+E compared to HP group. With this result we infer that the HP+E animals are able to improve the number of steps performed.

This kind of neurotoxic hemilateral lesion, provides a clear motor dysfunction behavioral response in RP-F direction of movement, otherwise LP-F parameter exhibits the compensatory mechanism relying on the non- lesioned (right) side. Initial time parameter has an analogous in PD, bradykinesia. This sign is evident in HP animals who display longer latency times in relation to the other groups. What is more, HP initial time in RP-F direction of movement is the highest, meaning less response in the lesioned group. Then, estrogen treatment improved this deficiency.

Stepping time was significantly different only in HP testing sequence. From this result we can conclude that compensatory mechanisms activate to perform forelimb movement, even though they can not restore the function in the lesioned animal. In the case of HP+E animals such difference is not evident, so there is a beneficial effect of estrogen that could be improving this pathological condition.

Step length is an indicator of step power. There is a tendency in HP+E animals to maintain the magnitude of movement near 49px (11.27 cm). In contrast, lesioned animals who did not receive estrogen treatment display a lower average energy, this fact can be interpreted as another estrogen compensatory mechanism.

To sum up, this software development and implementation is a worthy tool to *in vivo* infer on forelimb akinesia in an animal model of PD. By means of increasing the number of motor parameters assessed, there is a better picture of how the nigrostriatal system functions in normal and pathological conditions. In the same way, we obtain a new sensitive approach in the effect of estrogen as a neuroprotective drug in PD as it restores balance, mobility, speed and also diminishes bradykinesia.

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