Epilepsy & Behavior 122 (2021) 108166

Contents lists available at ScienceDirect

Epilepsy & Behavior

journal homepage: www.elsevier.com/locate/yebeh

Survival analysis of nonperiodic stimulation (NPS) performance

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ARTICLE INFO

Article history:
Received 11 April 2021
Revised 7 June 2021
Accepted 8 June 2021
Available online 31 July 2021

Keywords:
Epilepsy
Seizure
Electrical stimulation
Survival analysis
Kaplan-Meier
Logrank test

ABSTRACT

Electrical Stimulation (ES) of the nervous system is a promising alternative to treat refractory epilepsy. Recent developments in the area have led to a novel method involving a non-standard form of electrical stimulation with randomized inter-pulse intervals called non-periodic stimulation (NPS). Although it is an interesting approach, there is limited statistical proof to confirm its effectiveness. Therefore this brief communication presents a survival analysis of a pre-clinical trial to assess the significance of NPS therapy. The experiment comprised four groups of rats that have been compared: two with and two without NPS treatment. ES was applied bilaterally to the amygdala in animals subjected to the pentylenetetrazole continuous infusion (10 mg/ml/min) model, myoclonic or tonic-clonic generalized seizures were triggered. The Kaplan-Meier estimator was used to develop survival functions and the Logrank test was carried out to check the differences among groups. The first comparison was made between two groups of rats that developed generalized tonic-clonic seizures (GTC groups), those who received NPS treatment took longer to develop epileptic seizures. The logrank test proved statistical difference due to reaching a p-value of 7%. The second comparison was performed between two groups of rats that developed myoclonic seizures (MYO groups), and once again better survival probabilities were observed for the NPS group. The Logrank test revealed a p-value of 0.5% thereof. Thus, a survival analysis of NPS treatment proved effectiveness against seizures by promoting an anticonvulsant effect. By comparing the groups selected for this study, it was found that the NPS treatment yielded better results, mainly against myoclonic seizures.

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1. Introduction

Electrical Stimulation (ES) of the nervous system is a promising alternative to treat refractory epilepsy [1–3]. Based on the modern concept that the brain is a complex system and that synchronization is an emergent property resulting from the functional coupling of neuronal oscillators, epilepsy emerges as a hypersynchronization phenomenon [4,5]. The use of ES is an attempt to restore the brain's natural resonance dynamics [6]. In this context, a novel method has been developed which involves a non-standard form of ES with randomized inter-pulse intervals called non-periodic stimulation (NPS), firstly proposed by Cota et al. in 2009 [7]. De Oliveira et al. [3], Moraes et al. [5] and Cota et al. [6] discuss it in literature; however, a statistical analysis to evaluate its effectiveness has never been performed. Therefore, a Survival Analysis was carried out on account of being able to evaluate outcomes and aid pre-clinical decision making so as to anticipate progressions [8]. Park et al. [9], Dake et al. [10], Aderhold et al. [11] and Maharlouei et al. [12] conducted current medical studies using a survival analysis to evaluate treatments of diseases. Thus, the objective of this paper was to report the use of the survival analysis aimed to assess the NPS treatment effectiveness.

2. Methods

2.1. Kaplan-meier estimation and logrank test

The Kaplan-Meier estimation is a suitable alternative to measure the number of subjects that have lived for a specific time after treatment [13]. The statistical representation for the aforementioned definition is:

$$S(t) = P(T > t) = 1 - F(t) \quad (1)$$

where S(t) is the survival function, the complement of cumulative density function (CDF) F(t). ***T*** represents the ***time of death*** or ***failure***. Survival in this paper means not having developed myoclonic

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https://doi.org/10.1016/j.yebeh.2021.108166
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or generalized tonic-clonic seizures (events of interest). Therefore, according to Eq. 1, S(t means the probability of being seizure-free (behaviorwise) after time ***t***. The Kaplan-Meier method is an empirical non-parametric approach to estimate the survival function, mathematically defined as [14]:

$$\widehat{S}(t) = \prod_{j:t_j \leqslant t} \frac{n_j - d_j}{n_j} \quad (2)$$

where $\widehat{S}(t)$ is the Kaplan-Meier estimation function, t ($t_1 < t_2 < t_3 < t_k$) represents the times at which seizures were observed, d_j are the subjects affected by the event at time t_j (j = 1,2,3,. . .,k), and n_j is the number of subjects under risk prior to t_j. The Logrank test is a statistical tool aimed to evaluate Kaplan-Meier estimations by comparing them under a null hypothesis that there is no difference in population survival probabilities at any analysis time [15].

2.2. Animal experiments and database

The present experimental procedures with rats are described in full detail in De Oliveira et al. [16] and its theoretical background is reviewed in Cota et al. [17]. All database were acquired at the Laboratory of Neuroengineering and Neuroscience from the Federal University of São João Del Rei. A total of 49 Wistar male rats weighting 250–350 g from the main UFSJ vivarium and kept under a light-dark cycle of 12 h (lights on at 7 a.m.), food and water ad libitum, were divided into four groups, i.e., two control groups and two others that were submitted to the NPS treatment. Briefly, all animals underwent intravenous controlled infusion of convulsant drug pentylenetetrazole (PTZ), an unspecific GABAergic antagonist, at a rate of 1 ml/min and dilution of 10 mg/ml (thus 10 mg/min) as a model of acute ictogenesis and seizure induction. Infusion was interrupted at the onset of forelimb clonus (myoclonic seizures) in both NPS-MYO (n = 12) and MYO-CTRL (n = 14) groups. Similarly, PTZ infusion was interrupted only after occurrence of generalized tonic-clonic seizures in animals of NPS-GTC (n = 14) and GTC–CTRL (n = 9) groups. Although animals of these two last groups also displayed forelimb clonus (as well as several other convulsive behaviors) prior to generalized seizures, we did not include such time data points in the survival analysis of myoclonic seizures (Table 1 and Fig. 2A) in order to maintain the separation between groups and better correspondence to results of future analyses. While animals of NPS-MYO and NPS-GTC were submitted to concomitant NPS treatment, those of CTRL-MYO and CTRL-GTC did not and were used as controls for comparison. NPS was delivered as biphasic square pulses of constant current ranging from 150 to 400 μA (according to each animal's susceptibility) with 100 μs of duration each phase, average of four pulses per second, using an off-the-shelf equipment (8-channel stimulator with isolation units, models 3500 and 3800 from A-M Systems, Sequim, WA, USA). Bipolar electrodes made of twisted pairs of Teflon coated stainless-steel microwires (num. 7914, A-M Systems, Sequim, WA, USA) were surgically implanted in the amygdalae of both brain hemispheres with the aid of a stereotaxic apparatus (coordinates AP = 2.8 mm, ML = 5.0 mm from Bregma and 7.2 mm from Dura mater). Amygdala was chosen as the target for NPS given its central role in epileptic phenomena and its widespread mono or polysynaptic connectivity with structures in the forebrain, midbrain, and hindbrain. Considering the fixed rate of PTZ infusion and very small variability with no significant difference in animals' weight across groups (CTRL-MYO: 306±10 g; CTRL-GTC: 316±10 g; NPS-MYO: 311±6 g; NPS-GTC: 314±7 g), we measured the latency to trigger convulsive behavior as input parameter for the present survival analysis and as a means to assess NPS therapeutic effect. A summary of the experimental methodology is presented in the leftmost panel of Fig. 1. All procedures were previously approved by the Ethics Committee on Research Involving Animals (Comitê de Ética no Uso de Animais – CEUA) of the Federal University of São João Del Rei (UFSJ) (Protocol 31/2014). The procedures are in full accordance with international guidelines for the care of animals in research.

Table 1
Time registered for each rat from MYO-CTRL and NPS-MYO groups. The MYO column indicates whether the rat had myoclonic behavior (1) or not (0). CTRL and NPS represents the columns indicating to which group each rat belonged (control or treatment.

Rat	Time(s)	MYO	CTRL	NPS
1	82.10	1	Yes	–
2	91.20	1	Yes	–
3	41.50	1	Yes	–
4	94.10	1	Yes	–
5	79.50	1	Yes	–
6	68.00	1	Yes	–
7	86.40	1	Yes	–
8	61.50	1	Yes	–
9	92.00	1	Yes	–
10	85.30	1	Yes	–
11	92.30	1	Yes	–
12	53.10	1	Yes	–
13	86.00	1	Yes	–
14	88.00	1	Yes	–
15	82.60	1	–	Yes
16	88.90	1	–	Yes
17	82.40	1	–	Yes
18	42.40	1	–	Yes
19	102.40	1	–	Yes
20	128.80	1	–	Yes
21	106.90	1	–	Yes
22	102.70	1	–	Yes
23	118.80	1	–	Yes
24	94.10	1	–	Yes
25	100.90	1	–	Yes
26	90.70	1	–	Yes

2.3. Data analysis

A summary of the methodology applied herein is presented in Fig. 1. All analyses were developed using all data collected from the following groups of the pre-clinical trial: MYO-CTRL (n = 12), NPS-MYO (n = 14), GTC–CTRL (n = 9) and NPS-GTC (n = 14), involving a total of 49 rats. The first procedure was the tabulation of survival times observed for each group, namely, the time until the rats develop myoclonic and generalized tonic-clonic seizures (events of interest).

The second procedure was the Kaplan-Meier estimation so as to draw survival graphs for each group. After the plotting graphs for each group, they were compared accordingly to their respective groups – MYO-CTRL with NPS-MYO and GTC–CTRL with NPS-GTC – through the Logrank statistical test.

All survival and statistical analyses have been developed using Python programming through Lifelines package and a computer environment with 5th generation Intel i7 processor, 8 GB RAM and MacOS 10.14 operational system.

3. Results

The time at which each rat has developed seizures can be observed in Tables 1 and 2 - which has been recorded in seconds. By comparing the results in Table 1 (MYO-CTRL and NPS-MYO goups), it is possible to verify that there is a time difference between groups, which is higher on average for the NPS-MYO group, thus suggesting treatment efficacy against seizures. In Table 2 (GTC–CTRL and NPS-GTC groups) it can also be checked

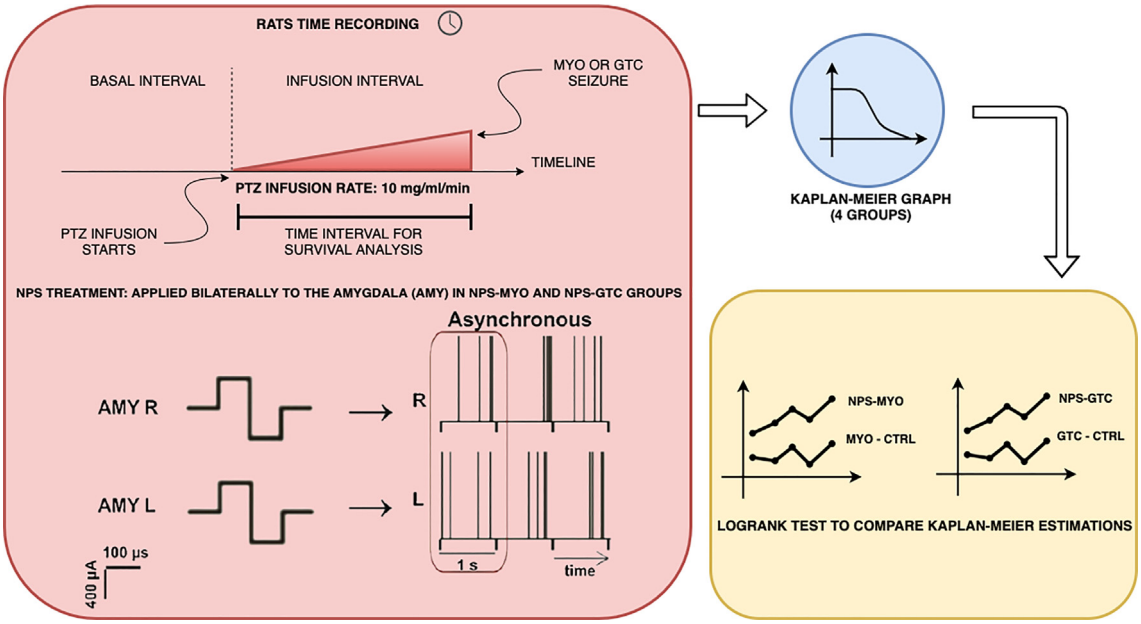

Fig. 1. Applied methodology. At first, the times at which all rats from the four study groups (MYO-CTRL, NPS-MYO, GTC–CTRL and NPS-GTC) developed seizure were recorded. Control groups underwent PTZ infusion to induce seizures and the times until observing myoclonic and generalized tonic–clonic behavior were recorded. NPS-MYO and NPS-GTC followed the same protocol as that observed for control groups, but the difference is that they were submitted to NPS therapy until the development of seizures. NPS treatment was applied bilaterally to the amygdala in animals subjected to the pentylenetetrazole continuous infusion (10 mg/ml/min) model. Afterward, the Kaplan–Meier estimator was performed for each group. Finally, the respective groups were compared through the Logrank test.

Table 2
Time recorded for each rat from GTC–CTRL and NPS-GTC groups. GTC column indicates whether the rat had generalized tonic–clonic seizure behavior (1) or not (0). CTRL and NPS represents the columns indicating to which group each rat belonged (control or treatment).

Rat	Time(s)	GTC	CTRL	NPS
1	127.50	1	Yes	–
2	133.60	1	Yes	–
3	109.00	1	Yes	–
4	146.00	1	Yes	–
5	147.70	1	Yes	–
6	140.10	1	Yes	–
7	139.40	1	Yes	–
8	159.70	1	Yes	–
9	166.00	1	Yes	–
10	189.20	1	–	Yes
11	130.60	1	–	Yes
12	198.20	1	–	Yes
13	122.40	1	–	Yes
14	145.70	1	–	Yes
15	148.10	1	–	Yes
16	163.90	1	–	Yes
17	163.00	1	–	Yes
18	135.60	1	–	Yes
19	168.00	1	–	Yes
20	114.00	1	–	Yes
21	129.60	1	–	Yes
22	212.60	1	–	Yes
23	107.20	1	–	Yes

that the NPS treatment achieved the desired effect by delaying the time length of rats' seizures.

The Kaplan-Meier estimation for MYO-CTRL and NPS-MYO groups can be observed in Fig. 2(a). There is a difference between control and treatment groups, i.e., the survival time for the NPS-MYO group is longer and survival estimation curves started to become different from around 40 s. The Kaplan-Meier estimation for GTC–CTRL and NPS-GTC groups can be observed in Fig. 2(a) and once again there is a noticeable difference between treatment and control groups. The survival graph is almost the same until reaching approximately 125 s, and then it becomes different, as it is higher for the NPS-GTC group. Graphically, it is possible to observe that the plot starts becoming different earlier for the NPS-MYO group.

The Logrank test revealed statistic of 3.36 and p-value of 7% when comparing the MYO control and treatment groups. Statistic was 8.59 and p-value of less than 0.5% when comparing the GTC control and treatment groups. Then, the results suggest a more pronounced difference between survival plots for the NPS-MYO group. As regards computational time, about one hour was spent to finish all Python analysis.

4. Discussion

The NPS treatment is based on the concept that the brain is comprised of oscillators that couple together by means of a natural synchronization mechanism [18]. Therefore, epilepsy seizure is a result of a hyper-synchronization phenomenon that can be suppressed by means of a desynchronizing temporal pattern of ES [19,18,20].

Thivierge & Cisek [21] contributed to the understanding of a temporal pattern in a network of neurons, thus suggesting the possibility of a non-periodic synchronization of heterogeneous networks of spiking neurons, which would aid in elucidating epilepsy dynamics and provide valuable information aimed to develop new types of treatments, such as a non-periodic stimulation.

Furthermore, Cota et al. [17] and De Oliveira et al. [16] performed pre-clinical trials with rats and achieved promising results from the NPS treatment. However, a more in-depth statistical analysis aimed to prove its effectiveness had never been performed. Here,the Survival Analysis in addition to the Kaplan-Meier estimator and Logrank hypothesis test carried out to compare survival plots further demonstrated that the NPS treatment is significantly efficacious.

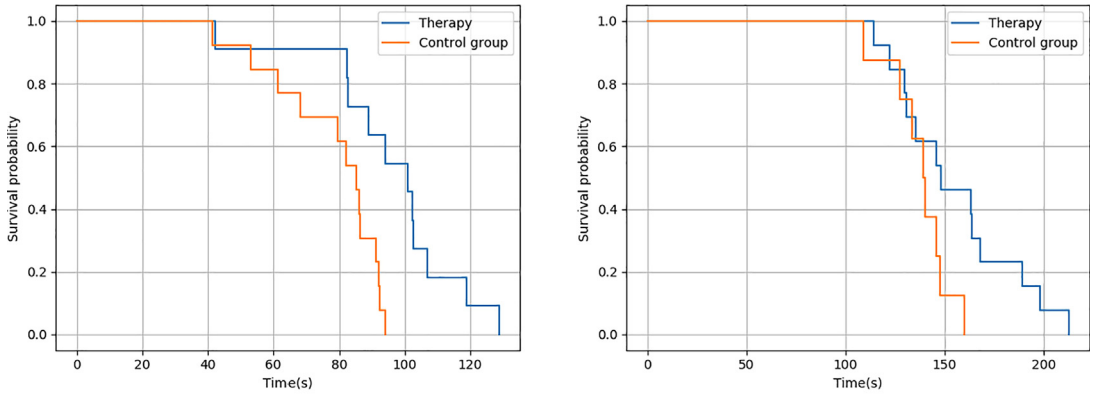

Fig. 2. (a) Kaplan–Meier estimation for MYO-CTRL and NPS-MYO. It is possible to observe that, for the NPS-MYO group, the survival probability graph is better than for the control group. It takes longer to observe the beginning of myoclonic behavior in the NPS-MYO group. (b) Kaplan–Meier estimation for GTC–CTRL and NPS-GTC groups. It is possible to observe a better survival probability estimation for the NPS group once more. It takes longer to observe the beginning of generalized tonic–clonic seizure in the NPS-GTC group. For both comparisons, the Logrank test is used to statistically prove the NPS treatment effectiveness.

The tables for treatment and control groups display time differences. Nonetheless, it was not possible to state that there is a significant difference among them. It was also not possible to suggest any difference between NPS-MYO and NPS-GTC treatments.

The Kaplan-Meier graph representing the survival estimations for each group more clearly indicates the difference between treatment and control groups, although it is still impossible to definitively assert that the NPS treatment has achieved a substantial difference. However, it can be observed that, for the NPS-MYO group, the survival graph becomes different from MYO-CTRL from around 40 s. The survival time observed is until over 120 s, and the survival probability for the control group is substantially different from that achieved by the treatment group, thus suggesting effectiveness. The same was observed when comparing GTC–CTRL and NPS-GTC groups, nonetheless, the difference between groups is closer to the survival time length. This indicates that the treatment is more efficient and presents better results for myoclonic seizure.

The Logrank test was useful to enhance the NPS treatment effectiveness. For MYO groups, a difference with a p-value of less than 0.5% was observed, but for GTC groups, difference with a p-value of 7% was observed. It is possible to conclude from the Logrank test that the NPS treatment increases the time to develop the epileptic seizure. Furthermore, it is again feasible and more effective when NPS is used to treat myoclonic seizures, which is evidenced by observing the Kaplan-Meier survival plots.

4.1. Strengths and limitations

In this study, we carried out the Survival Analysis using behavioral data only. On the other hand, electrophysiological data was of major importance to better understand epileptic phenomena and the underlying mechanisms of NPS. Such investigation is currently being carried out in the Laboratory of Neuroengineering and Neuroscience from the Federal University of São João Del Rei with promising results, a few of them already published [3]. Yet, the goal with this Brief Communication within the boundaries of a limited scope, which was to add further and more solid statistical evidence to that provided from the original study of NPS [7], which was carried out using only behavioral data.

Different values of the PTZ rate of infusion have distinct impacts on the latency to convulsive behavior results due to myriad factors (including pharmacokinetics), but many still unknown [22]. Yet, considering ethical issues on animal usage, the present infusion rate (10 mg/ml/min) has remained the same across many studies of the group and collaborators, including this Brief Communication.

It is also important to note that, although of great importance to drug assessment of novel treatments, the PTZ experiment is a model of controlled ictogenesis and acute seizures only, thus bearing considerable differences from clinical trial scenarios. A stronger extrapolation of the results to spontaneously occurring seizures in human patients would be better supported if such analyses were performed on data from animal models of epileptogenesis (e.g., fast amygdalar kindling, late-phase after pilocarpine-induced status epilepticus, etc.). However and for similar reasons, this is also beyond the scope of this Brief Communication.

5. Conclusions

The use of NPS treatment is a promising alternative for refractory epilepsy treatment. Although there are papers describing the approach and suggesting its success in assisting seizure suppression, a survival analysis to confirm its effectiveness has not been developed yet. To confirm the effectiveness of a new treatment, a survival analysis can be of assistance, given that it is widely known as a way to test new drugs and treatments in the medical area. Kaplan-Meier estimation graphically enhanced the clarity of the treatment results; nonetheless, it was not possible to state that there is a significant treatment efficiency, although it has been suggested that using the NPS to treat myoclonic seizure is more effective. The Logrank test evaluated and provided the statistical significance of the NPS treatment; moreover, it confirmed that its effectiveness is greater at treating myoclonic seizures. Therefore, using the survival analysis to evaluate NPS treatment provided a more complete overview and discussion about seizure suppression performance. Further work includes the assessment of results and applications of survival analysis in different experimental scenarios supporting a stronger extrapolation of the results to spontaneously occurring seizures in human patients.

Authors contribution

VHBT designed, drafted, and revised the manuscript. JCO designed and performed experiments and data acquisition. VRC supervised data acquisition and experiments, designed, drafted, and revised the manuscript. CDM designed, drafted, and revised the manuscript. No undisclosed groups involved in this study. All

co-authors have seen and approved the submitted version of the paper and accept responsibility for its content.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This work has been supported by the Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) [Grant No. APQ 02485-15] and it was partially supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001; FAPESP: 2014/50851-0 and CNPq: 465755/2014-3.

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