LONGITUDINAL DATA ANALYSIS REGARDING MEMORY LOSS (AMNESIA) IN RATS SUBMITTED TO CEREBRAL ISCHEMIA AND TREATED WITH FISH OIL

Matheus H. D.M. Ribeiro
Maringa State University, Maringa, Brazil - e-mail: dalmolinribeiro@hotmail.com

Humberto Milani
Maringa State University, Maringa, Brazil - e-mail: hmilani@uem.br

Isolde Previdelli
Maringa State University, Maringa, Brazil - e-mail: isoldeprevidelli@gmail.com

ABSTRACT

A study with repeated measures can be performed in different occasions. In many cases no knowledge of the methods associated with the analysis of correlated data may lead to erroneous results. In this study the aim is to, through the methodology of Mixed Models, verify anti-amnesic effectiveness of Fish Oil (FO) in animals with cerebral ischemia. Considering a model with random effect at the intercept, it was used the information criterion and restricted likelihood ratio test to choose the appropriate covariance structure. Statistical analyzes corroborate the biological evidence, statistically indicating the effectiveness of the treatment with fish oil in all therapeutic windows.

Keywords: Repeated measurements, Covariance structure, Latency, Fish Oil.

1. INTRODUCTION

Currently researchers from different areas of knowledge conduct studies to verify the effectiveness of treatments, drugs and products, in order to improve people's lives.

One of the main methods in statistics is modeling the behavior of one or more variable responses measured in units of one or more populations along some ordinate dimension (SINGER, NOBRE AND ROCHA, 2012). If the interest is to analyze the variable response of the problem once, there is a cross-sectional study. In this context, it is not possible to monitor the product performance or treatment over time, which is considered a disadvantage of the study.

On the other side if interest is to evaluate the behavior throughout time, there is a study with repeated measurements. In this case, one or more variables responses are measured repeatedly in the same unit of study.

A special study case with repeated measurements, are the Longitudinal Studies. The measurements are performed under different evaluation conditions and are arranged from time to time, respecting the analysis order. Data from Longitudinal Studies are characterized by temporal sequence of two or more observations in each individual.

These data types have a hierarchical structure, meaning that, repeated measurements for each individual have a structure of dependence with correlated errors (FAUSTO et al., 2008), and observations are independent among individuals. The assumption of correlated errors requires modeling of a covariance structure (KER E ANDERSON, 2003; FAUSTO et al., 2008).

According Diggle and Diggle et al., (1988; 2002) and Singer, Nobre and Rocha (2012) the covariance structures must accommodate three different sources of random variation, among which we can highlight the variation due to random effects, that can explain the correlation between the measurements and the variation from the measurement errors.

It was adopted the methodology of Mixed Models, intended to find answers to an experiment that proposes to evaluate the effect anti-amnesic of the Fish Oil in an animal model of cerebral ischemia.

2. MATERIALS AND METHODS

Figure 1 (a) shows the scheme on experimental planning to evaluate how varies the anti-amnesic effectiveness of Fish Oil (FO, 300 mg/kg DHA) on the basis of time, when treatment was initiated after ischemia (therapeutic window), more specifically, 4, 8 or 12 hours post-ischemia. Intact rats were trained in the aversive radial maze task (ARM, Figure 1b) for 10 consecutive days, and assigned to the different groups.
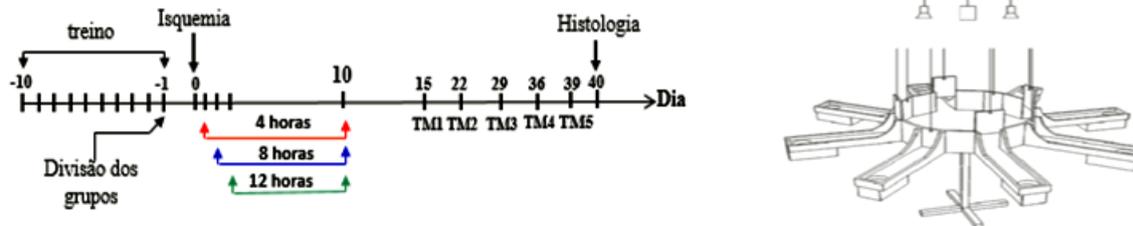
Figure 1. a) Experimental Planning and b) ARM - Radial Maze.

A post-workout or two days, patients were submitted to cerebral ischemia, and then immediately started treatment with FO, 4, 8 or 12 hours post-ischemia. After 15 days of ischemia, the animals were evaluated for their ability to remember the task learned during training, in other words, retrograde memory. Memory tests (MT) were applied once a week for 5 weeks. The following groups were tested: Sham (false-ischemic), Vehicle, FO 4 hours, 8 hours FO and FO 12 hours.

2.1 Behavioral Analysis

The operating principle of the ARM is based on the natural behavior of rat in to dodge an open and lit environment, to search for a narrow, dark place that offers you security. The ARM task consists on the animal to learn which of the eight arms the safe area is located at the end of one of the eight radial arms (hiding place). The ability to learn and memorize in the ARM is measured over several days of training (3 trials per day) and is measured by three parameters: (i) latency (ii) the number of errors of the reference memory, and (iii) number of errors of the working memory.

The latency expresses the time taken by animal to find the hiding place during each attempt at most 4 minutes. While exploring the maze, the animal makes mistakes when entering the false arms hiding places (7 in total). In each trial, the animal shall make a error of reference memory when entering for the first time in a false arm. If it returns to the previously visited arm is recorded then a working memory error. The reference memory is long term and is consolidated during the various trials and sessions, days of testing. And the working memory is short term and context-dependent, only relevant for a given time.

This study aimed to check if treated subjects (Groups FO) in different therapeutic windows, show improvement in their clinical condition compared to subjects who were not treated (Vehicle Group).

We present here only analyzes associated with the parameter latency. The results were obtained using the R software version 3.1.1 and SAS version 9.4.

3. RESULTS AND DISCUSSION

Of the steps involved in studies with longitudinal data it is possible highlight: The exploratory analysis of the data, determination of the covariance structure, adjustment and validation of the proposed model. The way to collect data provides us (a) if all the sample units are observed in the same instant, and (b) if the study is balanced regarding time, otherwise, if the observations are made irregularly, there is an unbalanced study (SINGER, NOBRE AND ROCHA, 2012).

In the present study the data are balanced regarding time and unbalanced according to the number of subjects per group. In order to evaluate the variability of the treatment groups, as well as the daily variability after initiation of treatment, it is possible to observe in Figures 2 (a and b) bellow.
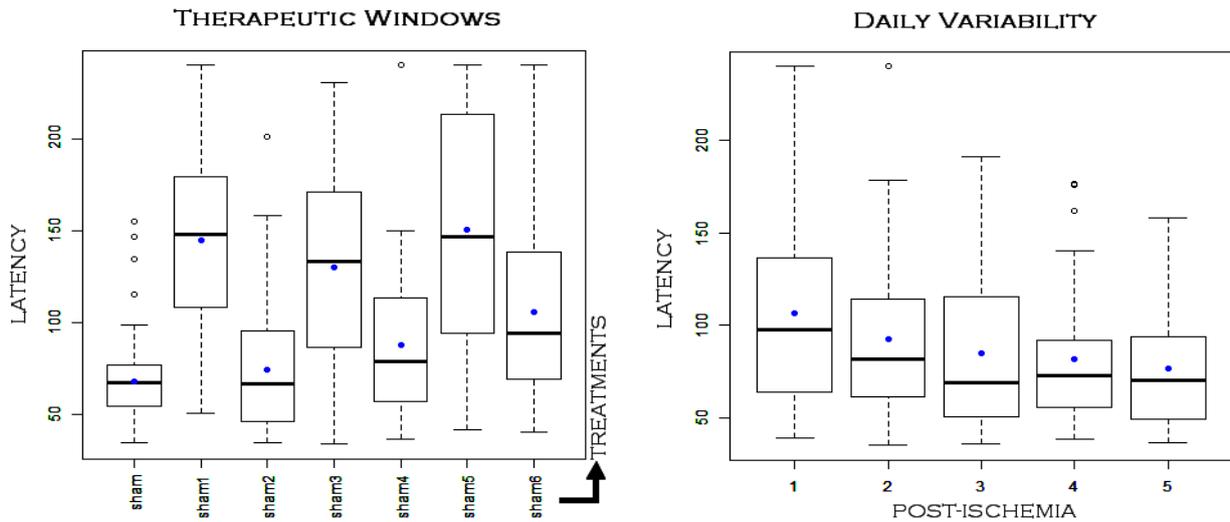
Figure 2. Data variability, a) treatments groups variability and b) post-ischemia daily variability.

To understand the variability of a data set is essential in statistical analysis. Based on the Figures 2 (a) and 2 (b), it is observed that the variance of the data for Group FO is lower compared to Vehicle Group, indicating the patient improves. Yet, as the days go by, it appears that the individual begins to reestablish its initial capacity.

Profiles plots are very important at the stage of exploratory data analysis. According to Singer, Nobre and Rocha (2012), they allow us to identify: sample intra-unit correlations, a possible heteroscedasticity and possible forms of the curve to be adopted to describe the data and outliers. Figure 3 shows the average latency parameter profiles for different groups in each of the therapeutic window studied.
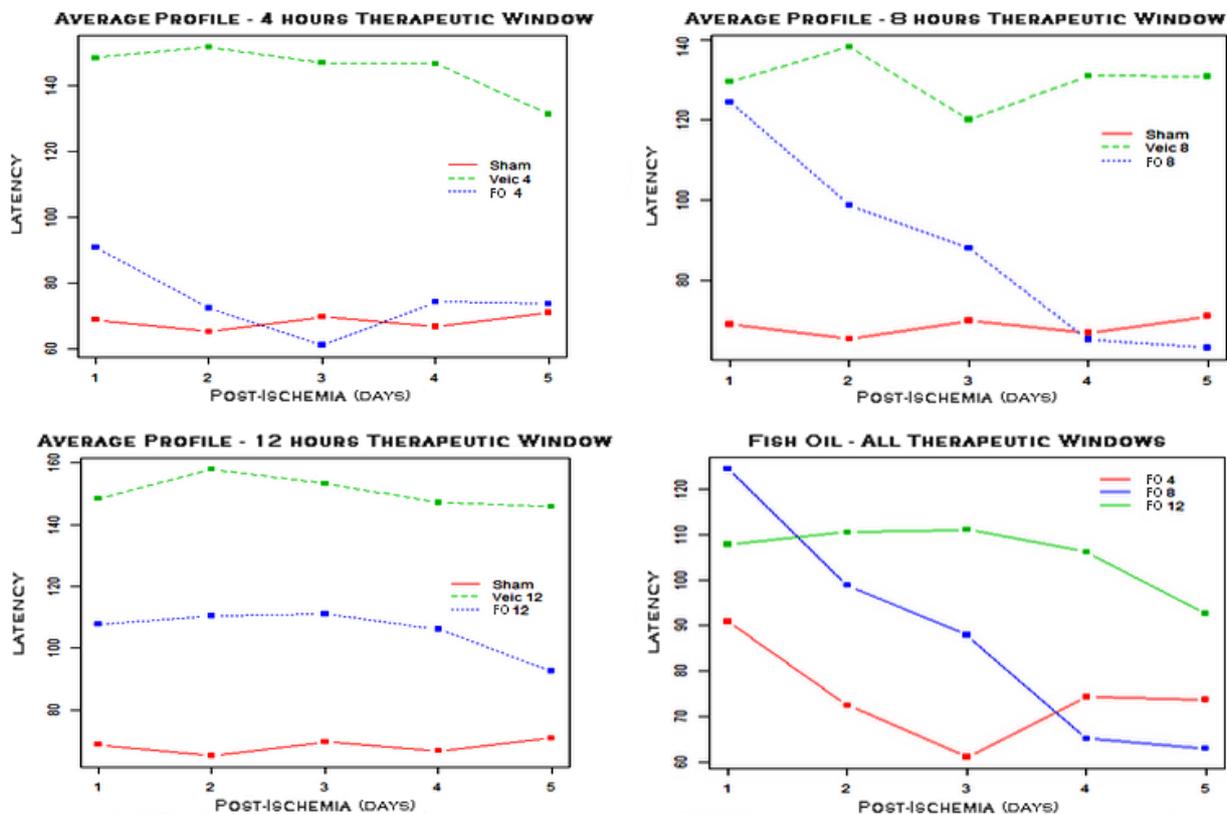
Figure 3. Average profiles, a) 4 hours post-ischemia, b) 8 hours, c) 12 hours and d) Fish Oil profile.

Through Figures 3 (a), 3 (b) and 3 (c), an effect of time - after the test days - only occurs in the diagram (b) for the FO Group. In the remaining, the latency does not change throughout the tests. Via the profile charts, it is possible to identify a possible linear relationship between the variables days and latency. Figure 3 (d) shows that the treatment started 4 or 8 hours after ischemia, provides rehabilitation on the individual (the lower the latency, the better the memory), indicating the importance of initiating treatment as soon as possible.

Prior knowledge about the correlation of data is the key to a later choice of the covariance structure. It allows us to identify patterns and possible trends that may be linked to the data. The covariance between repeated measurements is an important aspect that should be considered, in order to provide valid inferences for the parameters of effects model, used for modeling the data (FITZMAURICE, LAIRD AND WARE, 2004). The correlation matrices were evaluated for the three therapeutic windows studied. However, it is presented only the correlation matrix to analyze the relationship between the treatments with Fish Oil in three different moments corresponding to the beginning of the medication.

Table 1. Correlation matrix for the Fish Oil (FO) treatment in the therapeutic windows

	Day 1	Day 2	Day 3	Day 4	Day 5
Day 1	1	0.3757	0.2200	0.02040	0.1203
Day 2		1	0.5553	0.2397	0.1832
Day 3			1	0.4262	0.1928
Day 4				1	0.7733
Day 5					1

It can be clearly observed that there is a stronger correlation for observations made on days closer to each other than to those carried out in more widely spaced days. Yet, except for an observation, the correlation decreases gradually over time, which leads to a covariance matrix where the correlations are decreasing. A preliminary study was conducted, considering the data modeling used and the possible covariance structures. Thus, according to the structures found in the literature, it can be seen that the covariance structure that best accommodates the data is the Auto Regressive matrix (AR 1) or Compound Symmetric (CS) matrix.

The general form of a Mixed Model is provided by:

$$Y_i = X_i'\beta + z_i'\mu_i + \varepsilon_i$$

where Y_i is the response vector for i-th algorism; X_i' is the vector of covariates associated with fixed effects; z_i' is the vector of covariates associated with random effects (usually a subset of X_i'); β is the vector of fixed parameters; R_i and D are the covariance matrices of variance with $\varepsilon_i \sim N(0; R_i)$; $\mu_i \sim N(0; D)$ e ε_i e μ_i independents.

In order to the problem situation addressed, the effect model is given by:

$$y_{ijk} = (\mu + b_{i0}) + \gamma_j + \beta t_{ik} + \alpha(\gamma_j t_{ik}) + +\varepsilon_{ijk}$$

where y_{ijk} is the latency of the i-th animal, i=1,2,…,85, of the j-th treatment, j=1,2,3,4,5,6,7, observed at the k-th memory test (MT), k=1,2,3,4,5, μ is the general average; γ_j is the effect of the j-th treatment; t_{ik} is the the k-th MT; α is the interaction parameter for the j-th treatment and k-th MT, ε_{ijk} is the independent random error normally distributed with mean 0 and variance σ^2. In the analysis, it was considered a random effect on the intercept (b_{i0}), since each subject has a different "start".

The proper choice of the covariance structure is one of the main points in the data analysis. This choice can be made using the Restricted Likelihood Ratio Test (RLRT) where we compare nested models. When it has no nested structure, such as the AR frame (1) and CS, the choice can be made by observing the Akaike information criterion (AIC) and Bayesian information criterion (BIC), and by using waste analysis.

In a general context, there must be a match between the theoretical and practical results, regardless of the structure that is used.

Table 2 shows the information criteria when the structures AR (1) and CS are used, considering the data of patients treated with Fish Oil in the three therapeutic windows. By comparing the AIC and BIC information criteria for the adjusted structures, it is noticed that the lowest values are obtained when the AR matrix (1) is used. Thus, AR (1) it is considered as an appropriate structure. The estimated covariance structure is based on the data given by the side of Table 2 bellow.

Table 2. Information criteria

Obs	Description	AR (1)	CS
1	-2 Res Log Likelihood	1985.1	2001.0
2	AIC (Smaller is Better)	1993.1	2009.0
3	AICC (Smaller is Better)	1993.3	2009.2
4	BIC (Smaller is Better)	2000.0	2016.0

With $\phi = 0.4516$ and $\rho = 0.4516$, one standard error equal to 0.0697 and $\sigma^2 = 1517.70$ with a standard error of about 180.99. The estimations of the model parameters were obtained by the restricted maximum likelihood method, considering the treatment of eight hours as a reference and are shown in Table 3 bellow.

Table 3. Restricted Maximum likelihood Estimator of the parameters ($\alpha = 0.05$)

Parameter	Treatment	Estimation	Ep	GL	p-value	IC	
Intercept		135.73	13.0508	119	<.0001	109.89	161.58
Day		-15.47	3.7174	161	<.0001	-22.81	-8.13
DRUG	FO12	-20.56	18.4566	119	0.2675	-57.11	15.98
DRUG	FO4	-47.88	17.5701	119	0.0074	-82.67	-13.08
DRUG	FO8	-	-	-	-	-	
Day*DRUG	FO12	11.83	5.2572	161	0.0257	1.45	22.22
Day*DRUG	FO4	11.66	5.0047	161	0.0210	1.77	21.54

The marginal models based on the RMLE set of parameters are given by:

Latency for 4 hour window: $E(y_{i2k}) = (135,73 - 47,88) + (11,66 - 15,47) * t_{ik}$
Latency for 8 hour window: $E(y_{i3k}) = (135,73 - 15,47) * t_{ik}$
Latency for 12 hour window: $E(y_{i4k}) = (135,73 - 20,56) + (11,83 - 15,47) * t_{ik}$

Through residue analysis, the homoscedasticity and normality assumptions were satisfied. Treatments with FO started at different therapeutic windows were significant ($p < 0.05$). To verify the existence of differences between therapeutic windows, as well as in the treatment groups was utilized the Tukey test ($\alpha = 0.05$). When comparing the Sham groups, Vehicle and FO in the therapeutic window of four hours, the Sham and FO groups had no statistically significant differences ($p > 0.05$).

Nevertheless, when compared, FO and Vehicle groups were statistically different ($p < 0.05$). In the treatments started after 8 hours or 12 hours of the cerebral ischemia occurrence, all groups showed significant differences ($p < 0.05$). When comparing the treatments with FO in the therapeutic windows, FO 4 and FO 8 hours, had statistically significant differences ($p < 0.05$), which did not occur when comparing treatments FO 12 hours ($p > 0.05$). However, the absence of statistical evidence does not imply an absence of clinical evidence (ALTMAN AND BLAND, 1995).

4. CONCLUSIONS

Via the proposed methodology, it was found that the choice of covariance structure AR (1) suited correctly the variability of the experimental data.

The adopted mixed model was adequate to evaluate the data of the studied experiment. Statistical analysis corroborate the biological evidence, indicating the efficacy of the FO treatment. Regardless the beginning of the treatment, animals treated with FO has its cognitive ability restored, even partially.

Were identified statistically significant differences, when comparing the windows 12 hours for the groups treated with Fish Oil, and when comparing the Vehicle and FO groups. However, we must not ignore the absence of statistical significance in the remaining treatment windows.

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