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Applied Shared Log-Normal Frailty Cox-Proportional Hazard Model to Evaluating the Effect of Vitamin A on the Rat Passive Avoidance Memory

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Abstract: In this research, the Cox-proportional hazard model is used to investigate the effect of various values of vitamin A (3000, 4500 and 6000 IU kg⁻¹) and sesame oil on the passive avoidance memory of rats by shuttle box. Present results confirm that various values of vitamin A do not improve the passive avoidance memory of rats ($p < 0.05$). We found that the animals are clustered ($p < 0.001$) and applying shared log-normal frailty for clustering improves present results ($p < 0.05$) such that sesame oil improves the passive avoidance memory task ($p < 0.05$). Therefore we should consider clustering in the analysis of biological data or we should use cloned animals.

Key words: Passive avoidance, shuttle box, vitamin A, Cox-proportional hazard, clusters, log-normal frailty

INTRODUCTION

In many biostatistics studies, it is often desired to relate risk factors to the occurrence of a certain event. The Cox-proportional hazard model is widely applicable in the analysis of such data (Turcam and Rupp, 2000; Song *et al.*, 2003; Katz *et al.*, 2004; Costacou *et al.*, 2006). Moreover, subjects often are clustered and the survival times within a cluster tend to be correlated (Lin, 1993; Claus *et al.*, 1998; Bouhali *et al.*, 2007; Weycker *et al.*, 2007).

Clustering is an interesting area in the modeling of medical and biological experimental data where the subjects of study are often humans or animals. It is usual to select homogenous group of animals according to the standard protocols. But the animals usually are clustered because of unobserved biological variations between the animals or unmeasurable genetic factors lying behind the life process. However, in biological contexts, heterogeneity across subjects has usually been ignored (Grodstein *et al.*, 2003; Vakili *et al.*, 2004; Kumar and Gupta, 2002a; Greenwood *et al.*, 2007). It is important to include this heterogeneity in the statistical model in order to avoid misleading results.

Frailty model is useful to handling the within-cluster dependence (Vu and Knuiman, 2002; Kauermann *et al.*, 2008). In this model assumes that all the subjects within a cluster share a common unobserved characteristic, hence the hazard functions for the subjects within a cluster include a common frailty (Hougaard, 2000; Chuang *et al.*, 2005; Pankratz *et al.*, 2005) and Log-normal frailty model is one of popular frailty density function when using Cox-proportional hazard model (Hougaard, 2000; Kalbfleisch and Prentice, 2002; Vu, 2004).

Vitamin A is fat-soluble nutrients that exert various important roles especially during the early stages of life (Bates, 1983; Basu and Dickerson, 1996; Clagett-Dame and DeLuca, 2002; Berger *et al.*, 2008). Vitamin A is also fundamental for growth and development and it is essential to ensure a good functioning of the immune system of the young (Iwata *et al.*, 2004; Debier *et al.*, 2005). Moreover, vitamin A deficiency produce spatial learning and memory impairment (Cocco *et al.*, 2002; Etchamendy *et al.*, 2003; Hernandez-Pinto *et al.*, 2006; Oliveira *et al.*, 2007) and motor impairment (Cart *et al.*, 2006).

Sesame oil is one of the vegetables oil that consists unsaturated fatty acid and phosphatidylcholine (lecithin). Therefore it is effective on the decrease of cholesterol and improve spatial learning and memory in adult male mice, Moazedi *et al.* (2002).

Vitamin A (3000, 4500 and 6000 IU kg⁻¹) administered Intra muscular injection. The animals are injected for 6 days consecutively. Then all animals tested for a passive avoidance memory task by shuttle box. These groups have been compared with vitamin A sham group (sesame oil, 0.5 cc, injected group) and control group (any injection). The data is analyzed by one-way analysis of variances (ANOVA) (Adal, 2004). In this research the data reanalysis by using Cox-proportional hazard model with and without consider animals clustering.

MATERIALS AND METHODS

The young male NMRI rats (100±5 g and one month age) have been used. Animals were housed at constant temperature 23±2°C on a 12 h light/dark cycle and food and water available *ad libitum*. All experimental

protocols were applied in strict accordance with international guidelines regulating the use of animals for scientific purposes. This protocol has been done in department of Biology, Shahid Chamran University, Ahvaz, Iran, in 2004.

All animals tested for a passive avoidance memory task by the shuttle box, a Plexiglas box with a steel-rod floor (parallel rods, 0.25 cm in diameter set 0.8 cm a part), which includes two equal 30×20×20 cm connected parts, light part and dark part. The dark part is covered by Aluminum. There is a controlled door, 65×8 cm, between light and dark parts which is opened whenever it is needed. The shuttle box has been used to test memory activity and passive avoidance in animals (Lipman and Colowick, 1988; Kumar and Gupta, 2002a, b; Vakili *et al.*, 2004; Micale *et al.*, 2004; Pumo *et al.*, 2006; Greenwood *et al.*, 2007; Jin *et al.*, 2007).

One day before behavioral test, each rat was individually placed on the shuttle box and allowed to visit two parts freely for 3 min. After a rat had visited the box, it was removed from the box and returned to its waiting cage.

Each rat was gently placed on the light part 24 h later. When the rat leaves to the light part and placed its paws on the grid floor of dark part, the time is recorded (pre-test time). After 5 sec, it was taken an electric shock 1.5 mA (milli Ampere) without intermission for 2 sec. The rat removed from the box and returned to its home cage after 20 sec. The rats are clustered by the pre-test time such that the rat is clustered as an active rat if the pre-test time equal or less than 10 sec, otherwise it is clustered as an inactive rat.

Memory testing was performed 48 h later, in which each rat was placed on the light part again and the step into light part time recorded (test-time), in the absence of electric shock, with a step into time as passive avoidance behaviour. An upper cut-off of 300 sec was set (censored time). In this case, test-time was considered as 300 sec. It is cleared that the test time is not normal distributed and we cannot apply the one-way analysis of variance (ANOVA) to compare various groups of rats.

Drugs: The following drugs were used for passive memory: Vitamin A from GMB Co. (Germany), animal food from Pars animal food Co. (Iran). Rats were purchased from Razi institute (Karaj-Iran) and shuttle box with shock controlled was provided from Nortab electric Co. (Iran).

Experimental procedure: Thirty five animals were divided randomly into five groups so that each group includes seven animals. Vitamin A (3000, 4500 and 6000 IU kg⁻¹) (1 IU = 0.300-0.344 µg), values administered Intra muscular

(im) injection. Vitamin A for 3 days injected consecutively, the doses were adjusted in a way for each animal to receive a volume of 0.5 cc by adding sesame oil. These groups have been compared with vitamin A sham group (sesame oil, 0.5 cc (µL), injected) and with control group (no injection). All animal were tested, by the shuttle box, 24 h after last injection.

Data analysis: It is cleared that the normality of the data is not satisfied. The data is analyzed firstly by the Cox-proportional hazard model. In second time, the data is analyzed by the Cox-proportional hazard model with the log-normal frailty. In fact the pre-test time is considered for effect of the biological variations between the animals or genetic factors lying behind the life process of animals on the rats memory.

The analysis of the data was done by Splus 2000 statistical package. However these models are provided by any standard statistical package.

RESULTS

The frequency of observing event (enter into dark part) and censored (never enter into dark part) for two clusters, active and inactive is shown in Table 1. We found that there is no statistical significance between censored percentage of two clusters (p<0.05).

The results of the 95% confidence interval for the pre-test time for two cultures, active and inactive, are shown in Fig. 1. The average of pre-test time is 5.103 and 34.266 sec for the active and inactive clusters, respectively.

Statistical test confirm that the two clusters of rats are significantly different (p<0.001). Therefore the animals are clustered. Moreover, Fig. 2 shows the hazard of two clusters of rats, active and inactive, in fitting the Cox-proportional hazard model. As we can see the two clusters of animals have different hazard functions.

The results in Table 2 indicate that the sham group and different values of vitamin A groups did not statistical significance from control group (p<0.05) in the Cox-proportional hazard model. However, the sham (sesame oil) and 4500 IU kg⁻¹ value of vitamin A improve the passive avoidance memory of animals a little, with p-values 0.051 and 0.079, respectively, but there are not statistically significant (p<0.05).

Table 1: Frequency of event and censored in the two clusters of rats

Rat clusters	Event	Censored	Censored (%)
Active	13	3	18.80
Inactive	12	7	36.80
Total	25	10	28.60

Table 2: Results of fitting Cox-proportional hazard model with and without the log-normal frailty model

Model	Cox-proportional		Cox-pr. with log-normal frailty	
	$\hat{\beta}$ (SE)	p-value	$\hat{\beta}$ (SE)	p-value
Vitamin A Control	-	-	-	-
Sham	-1.586(0.811)	5.10%	-1.601(0.811)	0.048
6000 IU kg ⁻¹ A	0.841(0.558)	0.132	0.930(0.569)	0.1
4500 IU kg ⁻¹ A	-1.223(0.697)	0.079	-1.044(0.717)	0.15
3000 IU kg ⁻¹ A	0.331(0.562)	0.556	0.520(0.591)	0.38
σ (SE(frailty))	-	-	0.355	0.02
-2Log-Likelihood	138.257	0.00329	132.864	0.000457

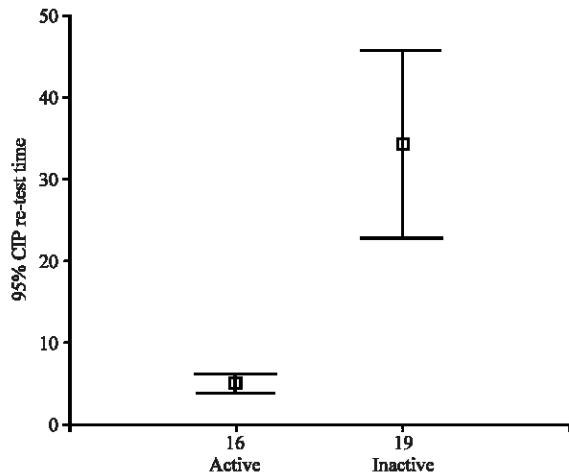


Fig. 1: 95% confidence interval for pre-test time for two groups of rats

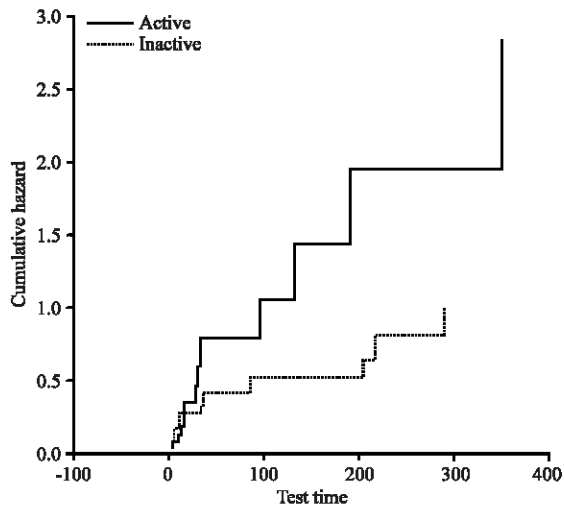


Fig. 2: Hazard function at mean of covariates

Adding the log-normal frailty to the Cox-proportional hazard model improves the results ($p < 0.05$) such that injected sesame oil (sham group) improves the passive avoidance memory of animals significantly ($p < 0.05$) as shown in Table 2.

DISCUSSION

Vitamin A is the generic term for a variety of substances including retinol, retinoic acid, retinyl esters as well as provitamin A carotenoids such as β -carotene. Retinol and its derivatives are only found in animal tissues whereas β -carotene is principally found in plants (Olson, 1984; Blomhoff, 1994; Basu and Dickerson, 1996). Vitamin A plays a critical role in vision, reproduction, immunity, cell differentiation as well as growth and development (Olson, 1984; Blomhoff, 1994; Basu and Dickerson, 1996; Napoli, 1999). More specifically, it is of utmost importance to allow successful gestation and proper offspring development (Thompson *et al.*, 1963; Clagett-Dame and DeLuca, 2002). It is also essential for the development and good functioning of the immune system (Blomhoff and Smeland, 1994; Semba, 1998). Vitamin A deficiency may induce foetal resorption, stillbirth and malformation (Bates, 1983; Basu and Dickerson, 1996; Clagett-Dame and DeLuca, 2002). It is also responsible for longer diseases, retarded growth and higher mortality in young mammals (Semba, 1998).

Experimental data suggest that oxygen free radicals are probably involved in the deterioration of cognitive processes. Vitamin A is powerful antioxidant and it protect tissues against the damage of the free radicals (Groff *et al.*, 1995; Ross, 1999; Bickford *et al.*, 2000; Alfors *et al.*, 2001). Previous studies have been found that vitamin A deficiency produce spatial learning and memory impairment in rats (Cocco *et al.*, 2002) and mice (Etchamendy *et al.*, 2003). It was also showed that vitamin A deficiency produces a severe deficit in spatial learning and memory which are linked to a proper hippocampal functioning (Hernandez-Pinto *et al.*, 2006).

We found that the various values (3000, 4500 and 6000 IU kg⁻¹), of vitamin A and sesame oil did not improve the passive avoidance of rats in the Cox-proportional hazard model without the frailty ($p < 0.05$). However, adding the frailty to the model to handling clustering improve the results and we can see that sesame oil improves the passive avoidance memory of rats significantly ($p < 0.05$). As resulted in Fig. 1, 2 and Table 2

showed the animals are clustered. Ignoring this heterogeneity across animals resulted in misleading statistical inference. Therefore this heterogeneity of animals should be considered to avoid misleading results. Otherwise we should to provide the biological homogeneous groups of animals (or cloned animals).

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