

The Effect of Repetitious Concussions on Cognitive Functions in Rats

Ratlarda Tekrarlayan Konküzyonların Bilişsel Fonksiyonlar Üzerine Etkisi

ABSTRACT

AIM: In this study, we aimed to investigate the effect of repetitious concussions on the spatial learning and memory function in rats.

MATERIAL and METHODS: 46 adult, male Sprague-Dawley rats were assigned to three homogenous groups by stratified random sampling according to their basal performance during the first four days of the Morris water maze (MWM), as Control (n=10), Sham (n=10) and Concussion (n=26) groups.

On the 12th day, the MWM experiment was repeated to evaluate the memory function. Rats in the concussion group were then subjected to the first head trauma. Some of the trauma-applied rats were sacrificed for histopathological examination 4 hours (n=4) and 24 hours (n=3) after the trauma. The spatial memory function of the rest of the rats was re-evaluated by MWM on the 20th day. Some of the rats were then subjected to the second head trauma (n=13), four of which were then sacrificed at the 4th hour, and three of which were sacrificed at the 24th hour. On the 27th day, six rats that had received single concussion and the remaining six that had received two concussions were re-evaluated for retention development by the third MWM experiment.

RESULTS: Escape latency curves showed a decrease in Sham and Control groups on the 20th and 27th days, and an increase in concussion group on the 20th day but no statistical significance was found between the groups.

CONCLUSION: Repetitious concussions did not significantly affect spatial learning-memory functions in rats, and may lead to a counter-adaptive mechanism against concussive injury.

KEYWORDS: Repetitious concussion, Head trauma, Concussion

ÖZ

AMAÇ: Bu çalışmada, sıçanlarda tekrarlayan konküzyonların uzaysal öğrenme-bellek işlevine olan etkisinin araştırılması amaçlandı.

YÖNTEM ve GEREÇ: Bu çalışmada kırk altı adet erişkin, erkek Sprague-Dawley sıçan kullanıldı. Sıçanlar, ilk 4 gün boyunca Morris su labirenti (MSL) aracılığıyla saptanan bazal uzaysal öğrenme düzeyleri (kaçış latansı) esas alınarak tabakalı randomize örnekleme ile homojen gruplara ayrıldılar: 1- Kontrol (n=10), 2- Sham (n=10), 3- Konküzyon (n=26). MSL deneyi 12. günde, retansiyon (bellek) gelişiminin test edilmesi amacıyla tekrarlandı. Ardından konküzyon grubundaki 26 hayvana ilk kafa travması uygulandı. Kafa travmasını takiben sıçanların dördü dört saat, üçü de 24 saat sonra sakrifiye edildiler. 20. günde MSL deneyi tekrar edildi. Daha sonra konküzyon grubundaki 13 hayvana ikinci kez kafa travması uygulandı. Bunlardan dördü dört saat, üçü de 24 saat sonra sakrifiye edildi. Ardından, tek konküzyon uygulanmış olan altı sıçan ile iki konküzyon uygulanmış olan altı sıçan 27. günde üçüncü retansiyon deneyi ile tekrar değerlendirildi.

BULGULAR: Kaçış latans eğrileri 20. ve 27. günlerde sham ve kontrol grubunda azalma eğiliminde iken 20. günde konküzyon grubunda artma eğiliminde idi. Ancak gruplar arasında istatistiksel olarak anlamlı farklılık saptanmadı.

SONUÇ: Uygulanan deneysel modele göre, tekrarlayan kafa travmaları sıçanlarda uzaysal öğrenme-bellek işlevini belirgin biçimde etkilememiştir.

ANAHTAR SÖZCÜKLER: Tekrarlayan konküzyon, Kafa travması, Konküzyon

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INTRODUCTION

Concussion is defined as a trauma-induced complex pathophysiological process affecting the brain that may be associated with rapid onset of short lived neurological dysfunctions such as transient loss of consciousness, retrograde amnesia, disorientation and attention deficits (4, 9, 19, 23, 33, 36).

Sports and recreational activities contribute to approximately 10% of all mild injuries with an estimated annual incidence of 300.000 cases (25). These individuals are at high risk of experiencing a second traumatic brain injury (TBI) (38) and the frontal lobe is found to be the most common site of focal lesions after mild traumatic injury (10).

Laboratory studies indicate that concussion may cause transient functional impairments due to pathogenic events such as excitatory amino acid release, alterations in regional cerebral blood flow and metabolism and ionic dyshomeostasis, and these events may sensitize the brain to a second TBI (15,18,22,39). It was also reported that players who sustained a concussion were three times more likely to have a second concussion (17) and athletes with multiple concussions might have cumulative effects (21).

Reproducible laboratory evidence on the duration of vulnerability of brain after a single concussion seems to be lacking (16), and there is still no consensus on the management strategies of concussions that occur during contact sports due to the lack of prospective data correlating outcome to the initial signs and symptoms of the injury (7,8, 12).

Although some authors suggested that multiple concussions lead to impairment in cognitive functions (13, 24, 26, 31, 32), Longhi et al, observed no deficits when the injury interval was extended to seven days (29).

The aim of the present study was to evaluate the effects of repetitious concussions on cognitive functions, in particular on the process of memory development and the extent of cortical and hippocampal cellular abnormalities in rats. Our objective was to investigate the above-mentioned functional effect, if any, by evaluating memory function in terms of retention development by using the Morris water maze, a tool for testing the spatial learning and memory function in rodents.

We hypothesized that repetitious concussions would diminish the deleterious effect of concussive injury on memory formation in rats.

MATERIALS and METHOD

Animals

The present study was started after having the project proposal approved by the Başkent University Local Ethical Committee on Research in Experimental Animals (approval date and number of the project; August 27, 2008 and DA08/16, respectively). Forty-six adult Sprague-Dawley rats (300 ± 50 g, male) were used in the study. All animals had free access to food (standard rat chow) and drink (regular tap water) throughout the study.

Acclimatization, preliminary training and initial experimental groups

After having the animals acclimatized to the environment by keeping them at the animal facility without performing any experimental procedure for 1 week, the experimental set up was introduced to the animals through a set of training sessions for three consecutive days. During this period, each rat was put in the Morris water maze (MWM) and allowed to swim for 15 seconds daily, as if it was a shortened version of the actual experiment, but with the maze lacking the escape platform. Data were neither collected nor evaluated during the preliminary training period.

Morris Water Maze (MWM)

MWM, an established method for evaluating spatial learning and memory development in rodents, was used to evaluate the effect of repetitious concussions on cognitive functions, particularly on memory development (34). A black-painted, water-filled, 120 cm width x 60 cm height polycarbon cylindrical tank placed in the middle of a cubical room (3x3x2.5 m) was used for the MWM experiments. A basic directional nomenclature was used to emphasize the localization of the walls, *i.e.*, South, North, East and West. Several permanent cues for orientation were permanently placed on the walls centrally. Two identical blue-colored rectangles of 35x50 cm size each, one yellow-colored circle of 48 cm diameter and three identical red-colored triangles of 44x38 cm size each were placed on the West, East and North walls, respectively. The South wall was intentionally kept blank. The temperature of the water in the tank was kept constant at $22 \pm 1^\circ\text{C}$.

The level of water surface was kept 10 cm below the top of the tank. An escape platform of circular shape (9 cm diameter) was placed 1 cm below the water surface at a certain location.

MWM procedure and evaluation of the spatial learning and memory process

Each rat was put in the water gently while facing the wall, at every direction each day, for four consecutive days. Each rat was given a time of maximal 120 sec at each direction to find the escape platform. The duration to find the platform was called the escape latency. If any rat succeeded to escape, it was allowed to stay on the platform for 10 additional seconds before taking it out of the tank, in order to eliminate the possibility of coincidental success and to help the rat to recognize the orientation cues. If the animal failed to find and step on the escape platform, it was guided to the platform by gentle touches and was let to stay there for 10 sec. The observer never entered the MWM room during the session unless the latter case happened, in which case he left the room after helping the rat step on the platform. The observation, data acquisition and evaluation procedure were performed by the customized software for animated image analysis via a computer connected to a webcam placed at the centre of the ceiling of the MWM room. The Mat-Lab based custom software for MWM experiments was developed by the Department of Biomedical Engineering at Baskent University, Faculty of Engineering, in order to evaluate the escape latency (sec), motor activity (swimming speed; cm/sec), cumulative distance swum (cm), and momentary distance to the escape platform (cm). The mean of the data from four directions each day was taken as the daily average of the above mentioned parameters and used to form cognitive function-time curves.

Experimental protocol

After having obtained the basal learning (escape latency) curves through days 1 to 4, the rats were initially assigned by stratified random sampling due to their basal cognitive performance to three groups; 1. Control ($n=10$), 2. Sham ($n=10$), and 3. Concussion ($n=26$), with lower inter-group but higher intra-group variations. On the 12th day, the MWM experiment was repeated to obtain retention (memory) data. The 26 animals in the Concussion group were then subjected to the first head trauma, four of which were sacrificed 4 hours, and three of which were

sacrificed 24 hours, respectively, after the trauma for histopathological examination. On the 20th day, the MWM experiment was repeated. Afterwards 13 animals from the Concussion group were subjected to the second head trauma, four of which were then sacrificed 4 hours, and three of which were sacrificed 24 hours, respectively, after the second concussion for histopathological examination. On the 27th day, six rats receiving a single concussion and the remaining six with two concussions were re-evaluated by the third retention experiment.

The Sham group was subjected to all of the procedures performed in the Concussion group except for the trauma process, while Control animals were kept naive to any procedure throughout the study.

Surgical Procedure

Rats were anesthetized with intramuscular ketamine 45 mg/kg and xylazin 7 mg/kg. The head of each animal was fixed in a stereotactic frame to prevent movement. A midline skin incision was performed to expose the calvarium. We used a silicon tip impactor driven by a pneumatic piston. The impounder was positioned at 90° and driven at 5.0 m/s to a depth of 3 mm farther than the zero point causing a non-penetrating concussive blow to the head with no skull fracture. After the compact brain injury the incision was closed with a 4.0 silk suture. The animals then were returned to their cages and allowed to recover from anesthesia. The neurological status of each animal was observed during the recovery.

Tissue fixation and Histopathology

Following the last controlled cortical impact brain injury, animals were reanesthetized intramuscular ketamine 45 mg/kg and xylazin 7 mg/kg and perfused intracardially with heparinized saline solution (1000 units/l) by transthoracic canulation of the left ventricle followed by fixative (4% paraformaldehyde in 0.1 M phosphate buffer, PH: 7.4). The scalp of each animal was opened under the operating microscope and skulls were observed for the possibility of any skull fracture. The brains were then removed and stored in a fixative for 4 h at 4°C, and then stored in 30% sucrose in phosphate-buffered saline (PBS) at 4°C. After sucrose infiltration, the brains were frozen in -20°C isopentane. Forty-micrometer thick coronal serial sections from 1.1

mm anterior to 2.8 mm posterior to the bregma were cut on a sliding microtome. Brain sections from all animals were stained by routine hematoxylin-eosin (H&E) procedure.

Statistical Analysis

All data were expressed as mean±S.E.M. Two-way ANOVA (time-procedure) was performed to determine the difference among groups. If any statistical difference was determined postHoc Bonferroni’s test was performed for further evaluation. Confidence interval was taken as 95% and P values <0,05 were accepted as statistically significant.

RESULTS

Cognitive performance

i. Basal learning and retention development

An improvement in learning performance, as expected, was apparently observed according to the to the escape latency values. Through days 1 to 4, the daily average values for escape latency were as follows: 39.6±2.3 sec, 19.8±1.5 sec, 17.1±1.5 sec and 13.4±1.3 sec, respectively (Figure 1). The average latency value obtained on the 12th day (13.2±1.1 sec) elicited no retention development, in comparison with the fourth day average (13.4±1,3 sec). We observed that escape latency curves showed a tendency to decrease in the Sham and Control groups on days 20 and 27, and a tendency to increase in the Concussion group on the 20th day but no statistical significance was determined between the groups (Figure 1). On

the 20th day the latency values of the groups were in a narrow range (14.0±1.5 sec; 13.2±2.5 sec; 12.3±1.1 sec), while on the 27th day the deleterious effect of repetitious concussion on retention became apparent, although none of the differences was significant (13.2±2.2 sec; 11.8±1.5 sec; 9.6±1.4 sec) (Figure 1)

ii. The effect of concussion(s) on the locomotor activity in the long term

Concussion(s) did not produce any significant effect on the parameters evaluating locomotor activity, such as swimming speed, cumulative distance swum and momentary distance to the escape platform (*data not shown*).

Histopathology

The formalin-fixed, paraffin embedded and H&E stained sections of brain tissues showed no specific morphological evidence of injury in all levels (Figure 2A). Only a small amount of edema was observed, particularly in the cortical areas and thalamus of animals subjected to cerebral brain injury (CBI) (Figure 2B).

DISCUSSION

The diagnosis and management of repetitious concussions have been debated in neurosurgery. Although some authors suggested that repetitious concussions may lead to cumulative effects (8, 13, 21, 24, 26, 31, 32 37), others reported that individuals may have an adaptive capability (20) and can be managed conservatively with rest (29, 35).

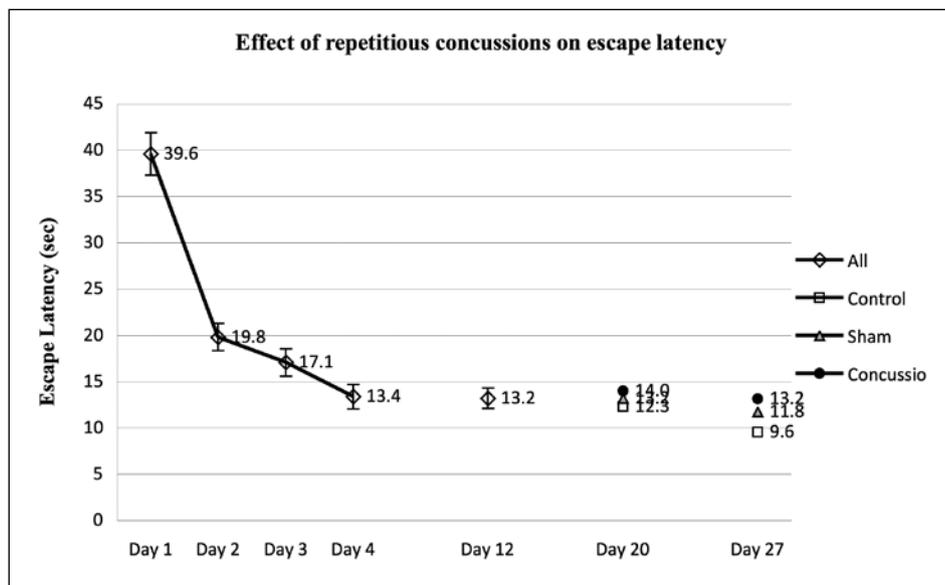


Figure 1: Effect of repetitious concussions on escape latency.

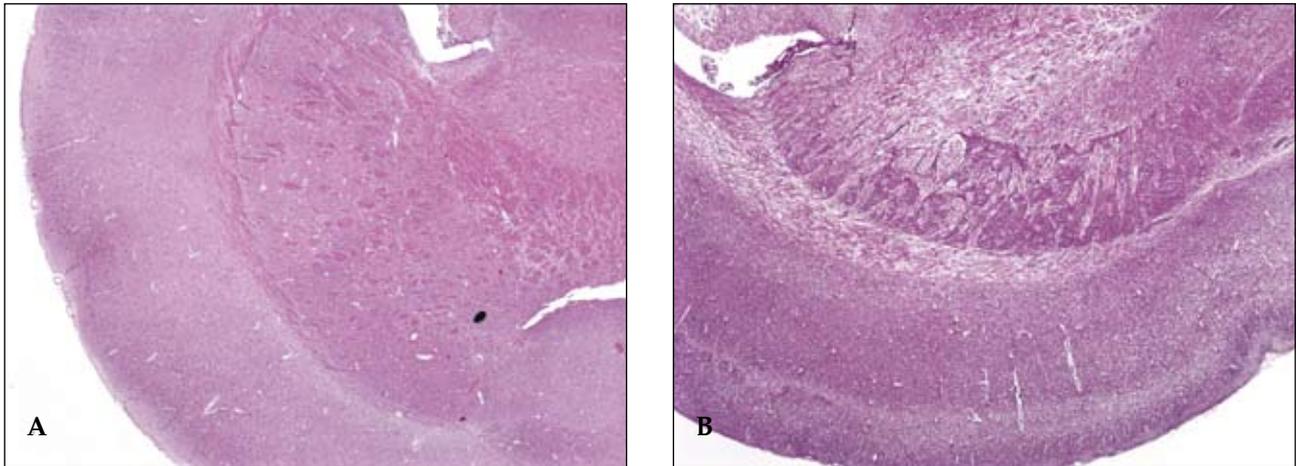


Figure 2: (A) Histopathology of brain tissues showed no specific morphologic evidence of injury at all levels (H&E, 40X), (B) A small amount of edema was observed particularly in the cortical areas and thalamus of animals subjected to CBI.

Laurer et al found that the brain was more vulnerable to second insult if the second injury occurred within 24 hours after the first mild head trauma (26). However, Longhi et al, reported that the vulnerability after the first cerebral brain injury (CBI) was transient and lasted one week after the first concussion and the occurrence of a second concussion does not produce additional cognitive and/or neurological motor alterations (29). Longhi et al. also stated that animals subjected to two seizures after repetitious CBI had significantly impaired cognitive functions such as learning, probably due to the additional damage as a result of excitotoxicity involving the hippocampus (29,40), but none of our animals had a seizure. DeFord et al. showed that four mild impacts to the brain, produced at intervals of 24 hours, led to learning deficits at 3 days after the last impact compared with mice receiving a single injury, suggesting that there is a cumulative effect of repetitious injuries on learning ability (11). Nevertheless, Hugh et al. reported that compact cerebral injury (CCI) in mice caused a marked decrease in immunolabeling for high molecular weight neurofilaments (NFH) in the ipsilateral cortex, hippocampal CA1 and CA3, dentate gyrus, and hilus within minutes and demonstrated that the partial recovery of NFH immunoreactivity following focal mild head trauma was apparent in the ipsilateral cortex and the hippocampal CA3 regions by 24 hours, indicating a potential adaptive capability of neurons (20). The recovery of neurofilaments (NF) after CCI is similar to observations in other animal models of traumatic brain injury of a transient loss of MAP-2 in vulnerable brain regions (5,14,28,29).

It is very difficult to describe the diagnosis and management of sport concussions due to the repetitious concussive injuries. The results in the few retrospective analyses are conflicting. While one study found that a history of two or more prior concussions predisposed athletes to significantly reduced cognitive function (7), another study found no postconcussion neurophysiological test differences between athletes who reported multiple prior concussions (30). Another study suggested that a single concussive brain injury (CBI) was not associated with cognitive impairment (29). Regardless of these conflicting data, the athlete must be completely symptom-free at rest or during exercise before resuming sports activity (8).

Our results proved that repetitious concussions produced at least at one-week intervals did not affect the baseline performance levels of the rats. No cumulative effect was observed as stated by Laurer, Longhi, DeFord and Hugh et al. as well. Performances following subsequent CBI were different from the initial performance levels obtained during the training period but lacking statistical significance.

All current concussion grading parameters evaluate the athlete's return to sport participation, at least in part, within the context of the number of prior concussions that the athlete has sustained (1,3,7,23), and guidelines have been developed to try to establish the safe interval between a concussion and the subsequent return to play (2,6,36). Pellman et al. recommended managing single and repeated concussions conservatively with rest and more than 50% of the players with repeated injuries returned

to play within a day (35). The latter is in accordance with the main finding of our study.

CONCLUSION

In light of the findings of the present study, we suggest that repetitious concussions do not affect cognitive functions significantly and may lead to a counter-adaptive mechanism against concussive injury in rats.

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