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Hippocampal Neuronal Damage in Rats Exposed to a Double Hit: Irradiation and Hyperthermia

Radyasyon ve Hipertermi Uygulanmış Sıçanlarda Hipokampal Nöronal Hasar

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ABSTRACT

AIM: In utero irradiation models induce diffuse neuronal damage. Experimental studies have shown that hyperthermia induced seizures are easily elicited and have high mortality accompanied by neuronal loss. Neuronal damage and loss are the results of cell death coupled with cortical development in altered cellular development. The aim of the study was to investigate the changes in hippocampus that was exposed to irradiation and hyperthermia.

MATERIAL and METHODS: Four groups were studied: 1) The irradiation group was exposed to 225 cGy irradiation on the 17th gestational day; 2) The hyperthermia group was exposed to hyperthermia on the 10th postnatal day; 3) The hyperthermia plus irradiation group was exposed to in utero irradiation and postnatal hyperthermia; 4) The control group was sham operated. Animals were examined 3 and 6 months later.

RESULTS: The hippocampus was atrophic with neuronal loss in CA regions and ectopic neurons were in irradiation group. Severe damage with the most atrophy was demonstrated in all regions of the irradiation plus hyperthermia group. In long term, damage was severe in all groups.

CONCLUSION: This study demonstrated more damage in hippocampi exposed to both irradiation and hyperthermia that may be taken as an evidence for the double hit hypothesis in the development of hippocampal damage.

KEYWORDS: Hippocampus, Hyperthermia, Irradiation, Neuronal damage, Rat

ÖZ

AMAÇ: In utero radyasyon yoğun nöronal hasarı indüklemektedir. Deneysel çalışmalar, hiperterminin nöbetlere neden olduğunu ve nöronal kayba bağlı olarak yüksek mortalite oranının olduğu gösterilmiştir. Nöronal hasar ve hücre ölümüne bağlı nöron kaybı, kortikal gelişim esnasındaki hücresel gelişim değişikliğinden kaynaklanmaktadır. Bu çalışmanın amacı, radyasyon ve hipertermi uygulanmış sıçanların hipokampusundaki değişiklikleri incelemektir.

YÖNTEM ve GEREÇLER: Dört deney grubu kullanılmıştır: 1) Radyasyon grubu: embriyonik 17. günde 225 cG radyasyon uygulanmıştır, 2) Hipertermi grubu: Postnatal 10. günde hipertermiye maruz bırakılmıştır, 3) Radyasyon ve hipertermi grubu: Hem radyasyon hemde hipertermi uygulanmıştır. 4) Kontrol grubu: Sıçanlar 3 ve 6 ay sonra histopatalojik olarak incelenmiştir.

BULGULAR: Radyasyon grubunda hipokampusun Ca bölgelerinde nöronal kayıp, ektopik nöronlar ve hipokampal atrofi gözlenmiştir. Radyasyon ve hipertermi grubunda ki tüm hipokampal bölgelerde yoğun atrofi ile birlikte yoğun hasar görülmüştür. Uzun dönemde bütün gruplarda hasar artmıştır.

SONUÇ: Bu çalışma, radyasyon ve hiperterminin hipokampus da yoğun hasar oluşturduğunu göstermektedir. İleriki çalışmalarda bu ikili modelin, hipokampal hasar oluşturmakta faydalı olacağını düşünmekteyiz.

ANAHTAR SÖZCÜKLER: Hipokampus, Hipertermi, Radyasyon, Nöronal hasar, Sıçan

INTRODUCTION

Certain developmental abnormalities of the cerebral cortex are closely associated with epilepsy in humans. The in utero irradiation model in rats shares many clinical and histopathological features with human malformation of cortical development that induce diffuse neuronal damage. Several animal models of cortical developmental malformations (CDM) have been established. Systematic long term monitoring has been performed only in the utero irradiation model (14). Architectural abnormalities were reported in neocortical and hippocampal areas in irradiated rats. It has been shown that migratory cells are most radiosensitive. Therefore, migration defects and neuronal damage were seen in the irradiated rat hippocampus (13).

Experimental studies have shown that hyperthermia induced seizures are easily elicited and have high mortality accompanied by neuronal loss in rats. It has been suggested in experimental and clinical studies that the underlying brain pathology could increase seizure susceptibility in early childhood (17, 10) and that this will generate seizures related to hippocampal sclerosis later in life. Hyperthermia has been widely used as a model for febrile seizures (16, 12, 15, 2). Hippocampal damage observed in young patients with neuronal loss and gliosis may be caused by hyperthermia. Hyperthermia-induced seizures occur more frequently in rats with malformations of cortical development (MCD) such as focal cortical dysplasia (10) and heterotopia (20).

It is currently unknown whether structural and physiological abnormalities in the model of in utero radiation with hyperthermia, as a second insult, are sufficient to produce a clinically relevant epileptic phenotype (19). In a short term follow up study CDM was demonstrated to increase the susceptibility to hyperthermia induced seizures in rats (10). However, a long-term follow up for the detection of histological changes in the hippocampus of rats exposed to in utero irradiation with hyperthermia has not been studied before.

In the present study, hippocampal damage induced by irradiation in utero and exposure to hyperthermia after birth, which is a double hit model in the developing brain, was designed to investigate subsequent histological changes in the hippocampus.

MATERIAL and METHODS

Animals and Experimental Design

Thirteen time-pregnant Wistar albino rats and their litters were studied. Rats were controlled daily for the presence of sperm cells by vaginal swabs, and the day at which insemination was detected was defined as embryonic day 0 (E0). Birth occurred on E21 or E22, and this date was designated as postnatal day 0 (P0). Rat pups remained with their mothers until they were weaned at P21. All animals were maintained on a 12-h light/ dark cycle with an average room temperature of 21± 3°C and with food/ water available ad libitum. The experimental study was approved of the animal care and Ethical Committee for Experimental Animals in Marmara University (23.11.2006-40.2005.mar).

The experimental design of the present study was as follows: (1) The irradiation group (n=8) in which the rats were only exposed to 225cGy radiation on E17; (2) The hyperthermia group (n=7) in which the rats were only subjected to hyperthermia on day P10; (3) The irradiation plus hyperthermia group (n=7) in which the rats were exposed to 225cGy irradiation on E17 and thereafter their litter were subjected to hyperthermia on day P10; (4) The sham-operated group (n=8) in which no irradiation and no hyperthermia was applied to age-matched control rats.

Exposure to Irradiation and Hyperthermia

In the irradiation experiments, the pregnant rats were anesthetized with 0.2 ml intramuscular ketamine and placed in the irradiator device. The animals were randomly divided into irradiation and sham-operated groups. In the irradiation group, abdomens of the rats were exposed to 225 cGy radiation on single fraction by using a cobalt 60 teletherapy system with SSD technique on E17.

In the hyperthermia experiments, the litters of rats which were exposed to irradiation in utero and another group of litters without exposure to irradiation were subjected to hyperthermia by heating them with a heating machine (where rectal temperatures reached 40°±0.1°C) on day P10. The duration of the hyperthermia sessions was 10±5 minutes until the rectal temperature reached 40±1°C and behavioral changes started.

Histopathological Procedures

Three or 6 months after irradiation, hyperthermia or both applications rats were deeply anesthetized with 50 mg/ kg ketamine and 12 mg/kg xylazine hydrochloride and then perfused through the aorta with a solution of 3% paraformaldehyde, 0.2% glutaraldehyde, 0.1% picric acid in 0.1 M HEPES (pH 7.4). After decapitation, the entire brain was removed and left in the same fixative at 4°C for 4 hours. The tissues were washed in 0.1 M HEPES (pH 7.4). Thereafter, the brains were serially sectioned in a coronal plane with a Leica VT1000R vibrotome at 25 µm and taken onto gelatin-coated slides. Cresyl violet staining was performed for neuronal cell counting and for measuring of hippocampal areas. Ten hippocampal areas from a mid-hippocampal region of each animal were calculated by theNIH Image Analysis Image J program. Stained sections from all animals were analyzed blindly.

Every fifth section (total of 10 sections from the mid-hippocampal region) was analyzed from the dorsal hippocampus of all groups for cell counting at the light microscopic level as described previously (8, 9). All neurons in the pyramidal cell layer of four hippocampal fields were counted in one section. These fields were: 1) CA1 (P1: stratum pyramidale); 2) CA2 (P2: stratum pyramidale); 3) CA3 (P3:

stratum pyramidale); 4) dentate gyrus (DG: granule cell layer). Hippocampal subfields in each section were counted under a Nikon Alphaplot-2 YS2 microscope at X40 magnification in 10^6 mm^2 field by using an eyepiece graticule.

Statistical Analysis

Data were expressed as means ± SEM. They were statistically evaluated using a one way ANOVA test and post-hoc Tukey test. Differences were considered to be significant when p was <0.05.

RESULTS

1. BEHAVIORAL OBSERVATIONS

a. Irradiation Group: Hyperactivity was observed. The baseline temperature was 34-35.5°C. There was no significant difference in baseline rectal temperatures between non-irradiated and irradiated animals.

b. Hyperthermia Group: Baseline temperatures of all animals varied between 34°-35.5°C in the hyperthermia groups. After the heating application, rectal temperatures reached 39°-40°C and then heating application was stopped. At this point seizures were observed. Seizures had usual characteristics of limbic onset involving behavioral arrest, staring (Racine stage 0), facial clonus, tail stiffness, wet-dog shakes, fore- and hind-limb clonus (Racine stage 3) in both groups. The animals that developed 8-10 min of tonic clonic seizures and terminated spontaneously were included in the study.

c. Irradiation plus Hyperthermia Group: All animals' baseline temperatures varied between 34°-35.5°C in the hyperthermia groups. The seizures characteristics of the irradiated rats were same as those of the hyperthermic group after heating application.

Mortality rates were 50% in the irradiation group, 31% in the hyperthermia group and 53% in the hyperthermia plus irradiation groups.

2. HISTOPATHOLOGICAL RESULTS:

a. Control Group:

Hippocampal Area: Hippocampal areas were calculated in similar sections of mid-hippocampus in all groups (Figure 1).

Histopathological Abnormalities: A regular morphology of hippocampal regions were observed (Figure 4A).

Neuronal counts of hippocampus:

Total number of pyramidal neurons in CA regions (Figure 2) and DG (Figure 3): Neuronal and granule cell numbers of control groups are shown in Figure 2, 3.

b. Irradiation Group:

Hippocampal Area: Hippocampal areas were significantly decreased in the irradiation group after 3 and 6 months compared to the controls. No significant difference was observed between the irradiation 3 and 6 month-groups (Figure 1).

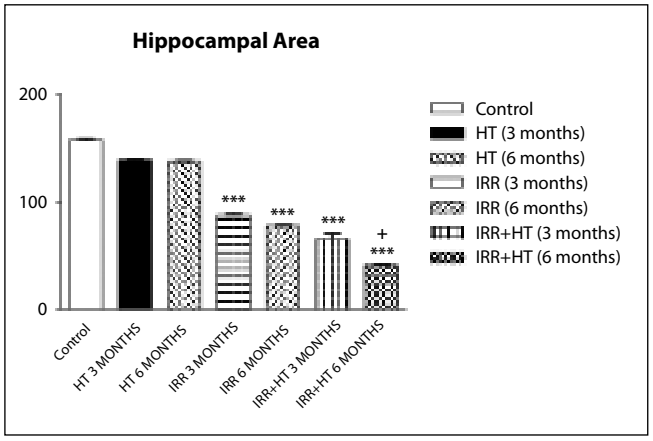

Figure 1: Graphic of hippocampal area (μm²). ****;** p<0,01, *****;** p<0,001 according to control group, **+;** p<0.05 according to 3 month after radiation and hyperthermia group.

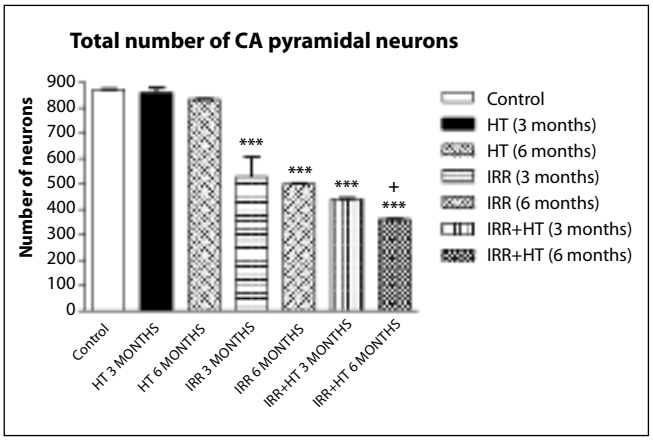

Figure 2: The density of total neurons (cells/10^6μm²) throughout CA regions. *****;** p<0,001 according to control group. **+;** p<0.05 according to 3 month after radiation and hyperthermia group.

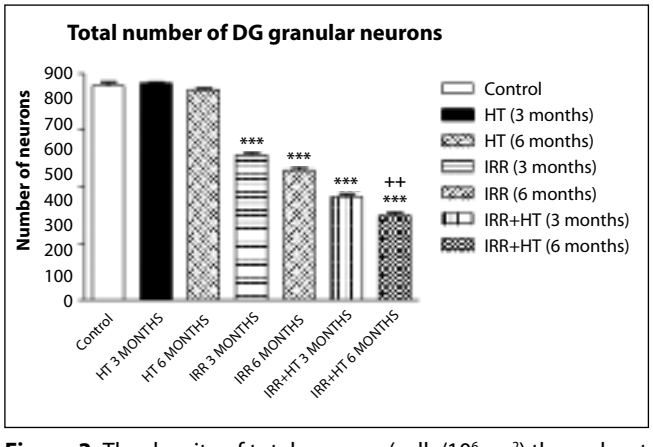

Figure 3: The density of total neurons (cells/10^6μm²) throughout DG regions. *****;** p<0,001 according to control group. **++;** p<0.01 according to 3 month after radiation and hyperthermia group.

Histopathological Abnormalities: Neuronal losses and ectopic neurons were seen in the CA1 and CA3 regions of the hippocampus at 3 months. Neuronal loss was severe and there were many ectopic neurons in the CA1 and CA3 regions. Neuronal dispersion was observed in the DG after 6 months (Figure 4A-K). Hippocampal atrophy was also severe after 6 months. Neuronal disorganization, neuronal loss and migration defects (Figure 4A-K) were seen in all litters that were exposed to irradiation.

Neuronal counts of hippocampus:

Total number of pyramidal neurons in CA regions (Figure 2) and DG (Figure 3): The total number of pyramidal neurons in the CA regions ($p<0.01$, Figure 2) and of granule cells in the DG ($p< 0.05$; Figure 3) were significantly decreased in the irradiation groups after 3 and 6 months compared to the controls, but were not significantly different between 3 and 6 months. The decrease in neuronal counts was correlated with the decrease in hippocampal area.

c. Hyperthermia Group:

Hippocampal Area: No difference was detected in hippocampal areas of the hyperthermia groups after 3 and 6 months compared to the controls (Figure 1). There was no significant variation in the hyperthermia group after 6 months compared to hyperthermia group after 3 months (Figure 1).

Histopathological Abnormalities: A small neuronal loss was seen only in the CA3 region in the hyperthermia group after 3 months and 6 months (Figure 4A-K).

Neuronal counts of hippocampus:

Total number of pyramidal neurons in CA regions (Figure 2): The total number of pyramidal neurons in the hyperthermia groups was similar that in the control group. Pyramidal neurons were more decreased in irradiation group compared to hyperthermia group after 3 and 6 months respectively.

Total number of granule cells in DG (Figure 3): There was no significant change in the granule cell numbers of the hyperthermia group after 3 and 6 months compared to the controls.

d. Irradiation and Hyperthermia Group:

Hippocampal Area: Hippocampal areas were significantly decreased in the 3-month and the 6-month irradiation plus hyperthermia groups compared to the control group. There was also a significant decrease of the hippocampal areas in the irradiation plus hyperthermia group after 6 months compared to irradiation plus hyperthermia group after 3 months (Figure 1).

Histopathological Abnormalities: Severe hippocampal atrophy was observed in the irradiation plus hyperthermia group after 3 and 6 months. Neuronal dispersion was observed in all CA regions. Severe neuronal losses and more ectopic neurons were present in both of the irradiation plus hyperthermia after 3 and 6 months groups. Migration defects, dispersion and neuronal losses were most extensive in the 6-month group of the irradiation plus hyperthermia group (Figure 5A-H). Moreover, the heavy decreases in neuronal numbers and hippocampal areas were remarkable after 6 months in the group of irradiation plus hyperthermia (Figure 1, 2, 3).

Neuronal counts of hippocampus:

Total number of pyramidal neurons in CA regions (Figure 2): The neuronal loss was greater in the irradiation plus hyperthermia group than in either of the irradiation and hyperthermia groups after 3 or 6 months ($p<0.001$). Furthermore, pyramidal neurons were significantly decreased after 6 months compared to 3 months in the irradiation plus hyperthermia groups. The decrease in neuronal counts was correlated with the decrease in hippocampal areas.

Total number of granule cells in DG (Figure 3): Decreases of granule cells were greater in the irradiation plus hyperthermia groups compared to the control groups and greater than both irradiation and hyperthermia groups. Granule cells were more decreased after 6 months compared to 3 months in irradiation plus hyperthermia groups ($p<0.01$).

DISCUSSION

Our study shows that exposure to in utero irradiation followed by hyperthermia on postnatal day 10 causes more degeneration in the hippocampus than does irradation or hyperthermia alone. This degeneration included neuronal loss in the CA regions except in CA2 and in dentate gyrus, as well as neuronal migration defects with ectopic neurons, neuronal losses and hippocampal atrophy.

Several animal models of cortical developmental malformations have been established, such as the focal freeze lesion model, in utero alkylating model and in utero irradiation model (7, 3, 18, 13). In the present study, we used the in utero irradiation model and treated the rats with 225 cGy radiation on day E17 to induce cortical and hippocampal disorganization. Different radiation sources have been used for in utero irradiation models but the results have been similar. Dosages in most studies ranged from 150 to 250 cGy (19). Many studies have suggested a differential effect of various doses of in utero radiation on the severity of the pathologic changes in treated rat brains and on the expression of in vivo epileptogenicity. However, the severity of cortical lesions is not always a predictor of epileptogenesis (13).

The timing of the exposure is a key factor in determining the histological results. Irradiation at E16 produces the most severe dysplasia of cortex with loss of lamination and loss of normal orientation of pyramidal cells and on E17 it produces inconsistent cellular death at all levels of the cortical mantle (4). Brains from adult animals that have been irradiated on E16 or E17 consistently show five major abnormalities: microcephaly, diffuse cortical dysplasia, neuronal heterotopia, focal areas of ectopic neurons in the hippocampus and agenesis or severe hypoplasia of the corpus callosum (6).

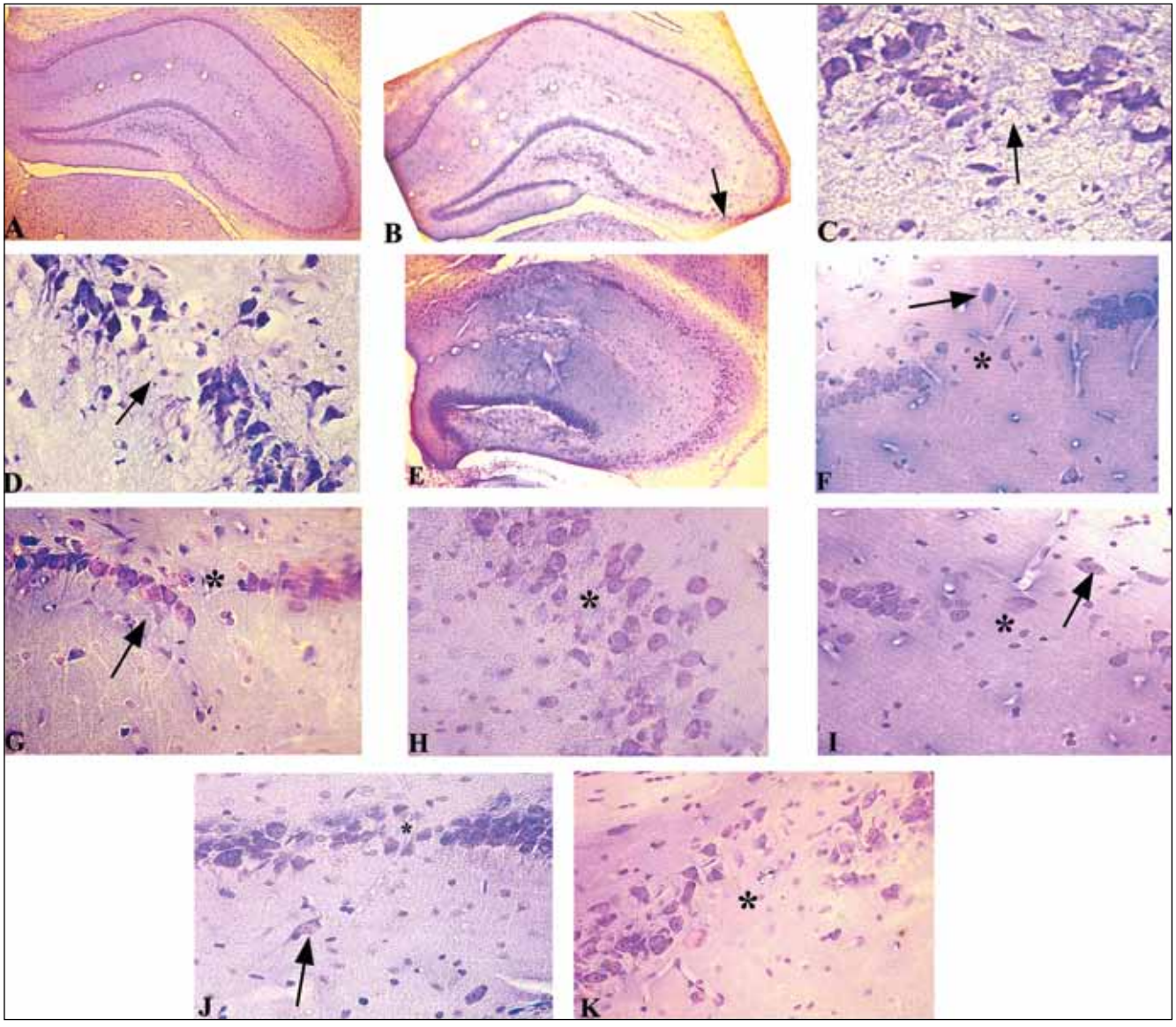

Figure 4: Regular morphology of control hipocampus **(A)**. Neuronal loss **(→)** in CA3 region in hyperthermia groups after 3 **(C)** and 6 months **(B, D)**. Atrophic hippocampus of irradiation groups after 6 months (E). Neuronal loss **(*)** and ectopic neurons **(→)**, in CA1 **(F, G)** and CA3 **(H)** region of irradiation groups after 3 months. Neuronal loss **(*)** and ectopic neurons **(→)**, in CA1 **(I, J)** and CA3 **(K)** region, dispersion in CA and DG regions **(E)** of irradiation groups after 6 months. Cresyl violet staining, original magnifications: A, B X40; C, D: X400; E: X40; F, G, H, I, J, K:X400.

Exposure of fetal rats to external irradiation produces diffuse neuronal heterotopia. These abnormalities are the result of radiation-induced cell death coupled with continued cortical development in an altered cellular environment (19). We mainly studied the effect of irradiation on hippocampal structures and found hippocampal areas to be atrophic in the irradiated animals. Total pyramidal and granule cell counts throughout the hippocampus were significantly decreased. Neuronal disorganization, neuronal dispersion, neuronal loss and migration defects were seen in all regions of the hippocampus. The hippocampi of irradiated animals frequently showed very focal areas of neuronal ectopia. This pattern of disorganization suggests that in these areas, some neurons that have failed to terminate migration at an appropriate level and migrated too far (17). Migratory cells are the most radiosensitive cells and relative sparing occurs in neurons that have already reached the cortical plate as well as in actively dividing cells in the ventricular zone (1, 4). Therefore, disorganization of both hippocampus and cortex increases with increasing radiation doses.

In this study we observed no significant difference in hippocampal areas of rats treated with hyperthermia only compared to control groups. However, irradiated rats which were also treated with hyperthermia showed significant

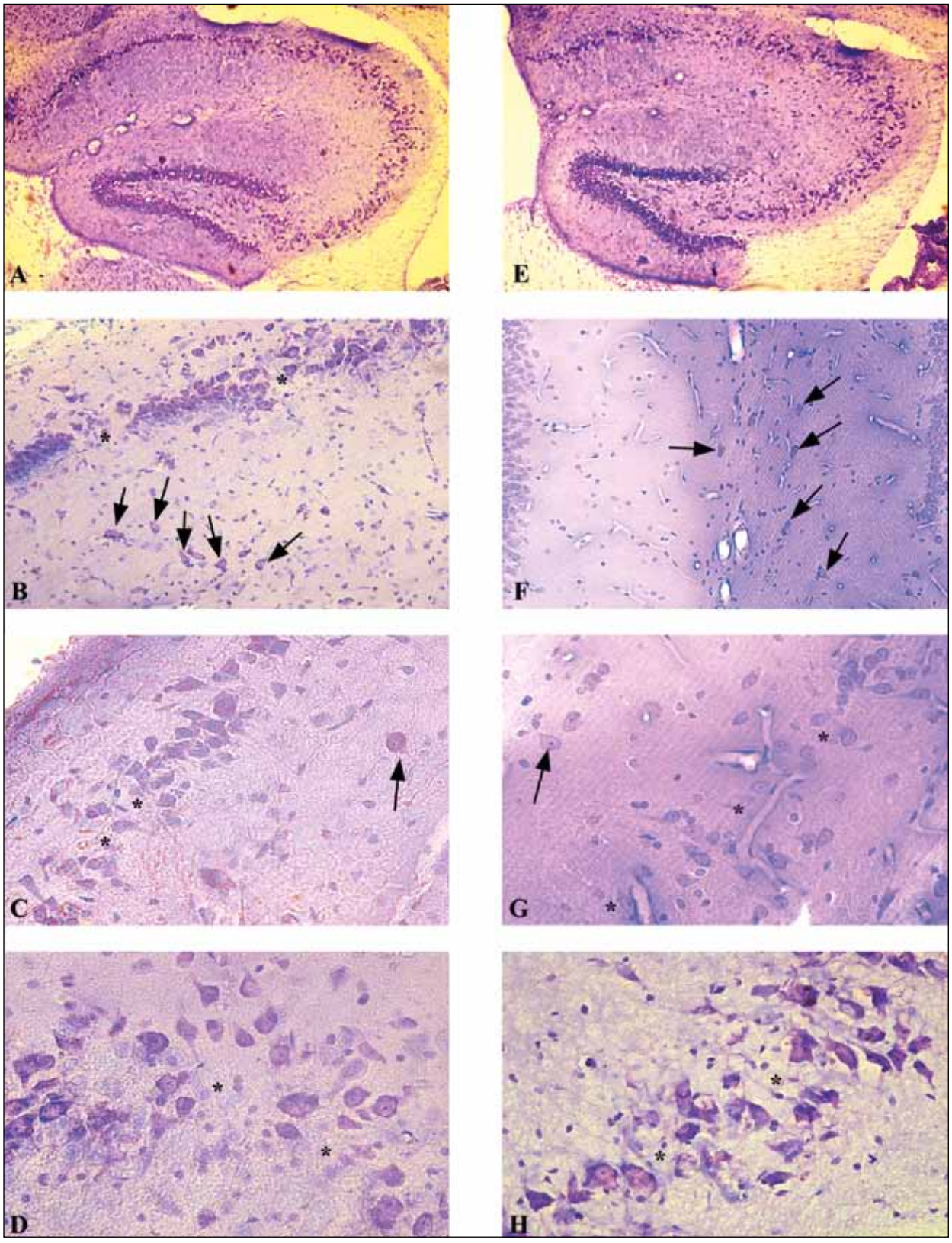

Figure 5: More severe hippocampal atrophy of irradiation and hyperthermia groups after 3 **(A)** and 6 months **(E)** after. Neuronal loss **(*)** and ectopic neurons **(→)**, in CA1 **(B, C)** and CA3 **(D)** region, dispersion **(*)** in CA regions **(A)** of irradiation and hyperthermia groups after 3 months. Neuronal loss **(*)** and ectopic neurons **(→)**, in CA1 **(F, G)** and CA3 **(H)** region, dispersion **(*)** in CA regions **(E)** of irradiation and hyperthermia groups after 6 months. Cresyl violet staining, original magnifications: A, E: X40; B, F: X200; C, D, G, H: X400.

hippocampal atrophy. This change was more remarkable after 6 months than 3 months suggesting a progressive continuing advance of the hippocampal damage produced by hyperthermia.

Clinical studies have shown that a family history of febrile seizures and neurodevelopmental abnormalities are risk factors for a first febrile seizure in children (5). However, in experimental animals it has been shown that behavioral seizures after prolonged hyperthermia occur significantly more often in rat pups with neuronal migration disorders than in normal control rats (10).

This study, comparing the effects of single injury with double injury and presenting long term changes as well, stresses the importance of preexisting lesions in the development of hippocampal injury in the presence of another risk factor such as hyperthermia. The progressive nature of this effect is an important consequence to be considered. However, these data are not sufficient to establish a clear causative relationship between developmental abnormalities and febrile seizures as there is also a well-known genetic background for febrile seizures. Nevertheless, the damage produced in pups exposed to both irradiation and hyperthermia suggests an additional effect, implying that children are more prone to develop cortical developmental malformations that may cause hippocampal damage/seizures.

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Disclosure Statement

None of the authors has any conflict of interest to disclosure.

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