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Original Article / *Özgün Araştırma*

Neuronal Rat Brain Damage Caused by Endogenous and Exogenous Hyperthermia

Ratlarda Endojen ve Eksojen Hiperterminin Sebep Olduğu Nöronal Beyin Hasarı

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Summary

Objective: Hyperthermia may induce pathologic alterations within body systems and organs including brain. In this study, neuronal effects of endogenous and exogenous hyperthermia (41 °C) were studied in rats.

Material and Method: The endogenous hyperthermia (41 °C) was induced by lipopolysaccharide and the exogenous by an (electric) heater. Possible neuronal damage was evaluated by examining healthy, apoptotic and necrotic cells, and heat shock proteins (HSP 27, HSP 70) in the cerebral cortex, cerebellum and hypothalamus.

Results: At cellular level, when all neuronal tissues are taken into account; (i) a significant increase in the necrotic cells was observed in the both groups ($p<0.05$), (ii) while exogenous hyperthermia did not affect apoptotic process significantly, endogenous hyperthermia led to a significant increase in apoptosis ($p<0.05$), whereas (iii) HSP 70 levels increased in neuronal tissues ($p<0.05$) in the endogenous hyperthermia group, but they decreased in the exogenous hyperthermia group ($p<0.05$). No statistically significant change was observed in HSP 27 levels in both of the groups ($p>0.05$).

Discussion: The neural tissue of brain can show different degree of response to hyperthermia. But we can conclude that endogenous hyperthermia is more harmful to central nervous system than exogenous hyperthermia. *(Turkish Journal of Neurology 2012; 18:11-6)*

Key Words: Hyperthermia, rat, brain damage

Özet

Amaç: Hipertermi, beyin dahil bütün organizmada patolojik değişikliklere sebep olabilir. Çalışmamızda endojen ve eksojen hiperterminin (41 °C) ratlarda nöronal etkisi gösterildi.

Gereç ve Yöntem: Endojen hypertermi lipopolisakkarit ile ve eksojen hipertermi yapay ısıtıcı ile oluşturuldu. Hiperterminin sebep olabileceği nöronal hasar, serebral korteks, serebellum ve hipotalamusda sağlam, apoptotic ve nekrotik hücreler ile, ısı şok proteinleri (Heat Shock Protein, HSP 27, HSP 70) incelenerek değerlendirildi.

Bulgular: Hücresel düzeyde, bütün nöronal dokular ele alındığında; (i) her iki grupta nekrotik hücrelerde belirgin artış görüldü ($p<0,05$), (ii) eksojen hipertermi apoptotic süreci belirgin olarak etkilemezken, endojen hipertermide apoptozis belirgin olarak arttı ($p<0,05$), (iii) HSP 70 seviyesi endojen hipertermide artarken ($p<0,05$), eksojen hipertermide azaldı ($p<0,05$). HSP 27 seviyesinde her iki grupta da belirgin bir değişim görülmedi ($p>0,05$).

Sonuç: Beyin nöronal dokularının hipertermiye cevabı farklı seviyelerde olmaktadır. Çalışmanın sonuçlarına göre, endojen hipertermi santral sinir sistemine eksojen hipertermiden daha çok zarar vermektedir. *(Türk Nöroloji Dergisi 2012; 18:11-6)*

Anahtar Kelimeler: Hipertermi, rat, beyin hasarı

Introduction

Body temperature is regulated by the thermoregulatory centre localized in anterior hypothalamus. Prostaglandin E2, which is produced by the effect of exogenous (like lipopolysaccharide) and endogenous pyrogens (i.e. IL-1, IL-6, IF-γ, TNF-α) on this centre, elevates the thermoregulatory threshold value and causes fever (1,2). In many organs, especially in brain, it causes an increase in the metabolism of cerebellum, cingulate gyrus, corpus callosum, thalamus and

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hypothalamus. Hyperthermia may cause permanent alterations within all body systems and organs especially in brain. Hyperthermia can cause deprivation of learning process, memory, motor and behavioural development (3,4). Hyperthermia has been reported to cause apoptosis. In addition, increased apoptotic process continues during hyperthermia (5,6).

Due to the evident difficulties in conducting studies while examining the effects of high fever and hyperthermia on child's brain, studies are limited to experiments using animal models. There are studies carried out using rat model reporting the effects of hypothermia and hyperthermia resulting in brain damage (7,8).

In this study, we tried to investigate and compare the effects of endogenous versus exogenous hyperthermia on brain through animal experiment model. It could be beneficial to understand possible neuronal damages induced by hyperthermia.

Materials and Methods

Selection of Animals for Experiment

We started the study having received the approval of the Ethics Committee and followed the guide for the care and use of laboratory animals. We used a total of 24 two-week old, female Wistar-albino rats, weighing 55±10 grams. By the time the experiment commenced, the rats had been housed in a temperature controlled room (22-25 °C) with a 12:12 light-dark cycle; water and food were given ad libitum.

The groups were formed by randomly distributed animals as described below;

Group I (Exogenous hyperthermia group with rectal temperature of 41 ºC; n=6) was exposed to external heat stress as described below.

Group II (Endogenous hyperthermia group with rectal temperature of 41 ºC; n=6) was exposed to internal hyperthermia by lipopolysaccharide (LPS).

Group III (Control Group, n=12) was composed of animals which were exposed neither to internal nor to external hyperthermia, and they were kept under normal conditions.

Generating Hyperthermia

The chambers - made of glass - in which rats were kept are 40 cm x 40 cm x 35 cm in size. Two holes were opened up for ventilation to get airflow on each facing wall. The roof of the chamber was designed in the form of a flat lid to place and take out the rats easily. A digital thermometer was attached to inside walls of the glass cage, 17 cm above the bottom, to measure the inside air temperature thus, enabling us to observe from the outside.

Infant rats to be used in the experiment were settled in groups into glass chamber (n=6). The inner temperature of the chamber was set at room temperature. Rectal temperatures of the animals were monitored with a rectal probe.

Subsequently, in the endogenous hyperthermia group, 250 microgram/kg intraperitoneal lipopolysaccharide (LPS) (Escherichia coli Serotype 0127:B8 lipopolysaccharide Sigma, USA) was administered to rats except for the control group and normal saline solution at the same volume was administered to the control group. About 2 to 6 hours later after the administration of LPS, rats had a temperature of 41 °C. When the intended body temperature was achieved, animals were exposed to hyperthermia for 60 minutes while their temperatures were being checked at 10 minute intervals.

In the exogenous hyperthermia group, lid was located on the top of the chamber for an easy access. An adjustable heat regulator was located underneath the lid. Two digital thermometers were placed on the inner surface of two walls facing each other, allowing measurement of heat from the outside. Animals were placed in the chamber in groups (n=6), exposing them exogenous heat stress. Before placing a new group of animals, chamber was cleaned and its temperature was checked and adjusted as desired. While temperature was set at 48 °C, inside temperature of Hyperthermia Induction Chamber (HIC) was found to be 45 °C. Rectal temperatures of animals were monitored using rectal probes as described earlier. When desired temperature was achieved, animals were exposed to this constant temperature for 60 minutes.

Finishing the Experiment

At the end of the experimental procedures, all rats were decapitated. Head region were dissected rapidly and their brains were extracted. Hypothalamus, cerebral cortex, corpus striatum and cerebellum were taken out carefully. Half of hypothalamus and cortex and the whole cerebellum were kept in formaldehyde for subsequent pathologic examination.

Histopathologic and Immunohistopathologic Examinations

The brains were fixed in 10% formalin and embedded in paraffin. Coronal slices were obtained from cerebral cortex, hypothalamus and cerebellum according to the stereotaxic atlas of the developing rat brain. Neuropathological evaluation was performed using the hippocampus, cerebral cortex and cerebellum on the coronal plane. After slices were obtained with microtome, samples were stained with haematoxylin-eosin (H&E). Stained preparations were examined under light microscopy (x400). Possible neurological damage findings were evaluated according to neuroanatomical regions. For this purpose, healthy and necrotic cells were counted at six different areas in cerebral cortex, cerebellum and hypothalamus. Subsequently, the means obtained from six different areas were used. Scores were obtained from counting healthy and necrotic neurons, and their ratio were used to assess brain damage.

Sections 20 mm in thickness were obtained and stained with (H&E) and with TdT-mediated dUTP biotin nick-end labelling (TUNEL). For TUNEL staining and evaluation of apoptosis, paraffin-embedded brain sections were deparaffinized in two cycle of xylene for 5 minutes each, then hydrated in 100, 95 and 70% ethanol. After incubation with 20 mgml-1 proteinase K for 5 min, a modified terminal deoxynucleotidyl transferase-mediated UTP nick end labelling (TUNEL) method was applied using an in situ apoptosis detection kit (ApopTag, Oncor, USA). Briefly, this includes immersion in equilibration buffer for 10 min, application of terminal deoxynucleotidyl transferase (TdT) and dUTP-digoxigenin at 37 °C for 1 hour,

followed by stop/wash buffer at 37 °C for 30 minutes incubation with antidigoxigenin for 30 minutes, and visualization with 0.05% diaminobenzidine tetrachloride and 0.02% H_2O_2 in 50mM Tris- HCl buffer. Counterstaining was carried out with methyl green. Slices were treated similarly but in the absence of TdT enzyme, digoxigenin-dUTP, or anti-digoxigenin antibody were included in the study as negative controls. Apoptotic neurons were determined using the image analysis system (Zeiss Vision KS400 version 3.0) by systematically randomized sampling through a 100_Nikon oilimmersion lens. The percentage of apoptotic neurons was calculated from apoptotic neurons to all neuron ratio in all brain areas, and scored as follows: 0=no apoptotic cells, 1=less than 25%, 2=25-50%, 3=50% and over.

Two heat shock proteins (HSP), HSP 27 and HSP 70, were used so as to evaluate the damage in the same parts of brain regions. For this purpose, HSP 27 and HSP 70 antibodies purchased from Lab Vision (Fremont, CA, USA) were used as immunohistochemistry. Obtained slices were marked with HSP 27 Ab-1 (Clone G3.1, Labvision, USA) and HSP 70 Ab-3 (Labvision, USA) antibodies.

Histopathological hyperthermic damage assessment subgroups were categorized as follows: healthy cells, necrotic cells, HSP 27 cells, HSP 70 cells, apoptotic cells.

Statistical Analysis

Statistical analysis was carried out by using the SPSS for Windows, Ver.12 (SPSS Inc. Chicago, IL, USA). The data obtained are expressed as mean±SD. Two-way ANOVA test was used for comparison among the groups, and $p<0.05$ was considered to be statistically significant.

Results

Behavioral Changes Observed in Animals

Initially there was an increase in the movements of rats in both groups. Rats were climbing upon each other, laying down on their backs or on their sides, or trying to stand up on their hindfeet. Later on their movements slowed down considerably.

The first clinical variation between the groups was that, endogen febrile convulsion rate in endogenous hyperthermia was significantly higher than exogenous febrile convulsion (41%- 33%).

Cerebral Cortex:

When only endogenous hyperthermia group was compared with control group, healthy cell count decreased significantly and necrotic cell count increased notably ($p<0.05$) (Table 1, Figure 1,4).

There were significant increases in apoptotic cell count in cerebral cortex in both of the groups ($p<0.05$) (Table 2, Figure 2,5). HSP 27 and HSP 70 levels were affected in neither endogenous nor in exogenous hyperthermia groups (Table 3, Figure 3).

Cerebellum

The necrotic and apoptotic cell count were significantly increased in both groups. There was an increase in HSP 27 and HSP 70 rates in the exogenous hyperthermia but there was no change in the endogen hyperthermia group (Table 1, 2, 3).

Hypothalamus

While healthy cell count and HSP 27 and HSP 70 rates were not affected in the endogen hyperthermia group, apoptotic cell count increased to a large extent. There was a significant decrease in the healthy cell count and no difference was observed in the necrotic, apoptotic cell count and HSP 27 and HSP 70 rates in the exogenous hyperthermia group.

All Neuronal Regions

The necrotic cell counts increased significantly in both groups, but the healthy cell counts did not change to a great extent. The HSP 70 rates increased significantly in the endogenous hyperthermia in all neuronal tissues; whereas they decreased in the exogenous hyperthermia group. On the other hand, the HSP 27 rates were not affected. While endogenous hyperthermia facilitated the apoptotic process, exogenous hyperthermia had no significant effect on apoptosis.

Discussion

Although the metabolic, immunologic and other side effects of fever can be tolerated by normal children to an extent, these effects can be worsened and may be dangerous under certain clinical situations (3). Moderate increase in fever may result in strong immune response following an increase in

Table 1. Effects of endogenous and exogenous hyperthermia on healthy and necrotic cell numbers compared to control group

Study Groups	Brain Region	Healthy Cell Number *	Necrotic Cell Number *
Endogenous hyperthermia	Cerebral cortex	Decreased	Increased
	Cerebellum	Unvaried	Increased
	Hypothalamus	Unvaried	Unvaried
	All brain region	Unvaried	Increased
Exogenous hyperthermia	Cerebral cortex	Decreased	Unvaried
	Cerebellum	Unvaried	Increased
	Hypothalamus	Decreased	Unvaried
	All brain region	Unvaried	Increased

*Increased/Decreased: $p<0.05$; Unvaried: $p>0.05$.

Table 2. Effects of endogenous and exogenous hyperthermia on neuronal apoptotic process compared to control group

Study Groups	Brain Region	Apoptotic cell levels *
Endogenous hyperthermia	Cerebral cortex	Increased
	Cerebellum	Increased
	Hypothalamus	Increased
	All brain region	Increased
Exogenous hyperthermia	Cerebral cortex	Increased
	Cerebellum	Increased
	Hypothalamus	Unvaried
	All brain region	Unvaried

*Increased/Decreased: $p<0.05$; Unvaried: $p>0.05$.

the production of interferon, lymphocyte transformation and leukocyte migration. On the other hand, immune response is suppressed by fever at 40 °C or so. Increase in every 1 °C in fever causes 10-12% increase in basal metabolism.

Table 3. Effects of endogenous and exogenous hyperthermia on HSP 27 and HSP 70 levels compared to control group

Study Groups	Brain Region	HSP 27 Levels *	HSP 70 Levels *
Endogenous hyperthermia	Cerebral cortex	Unvaried	Unvaried
	Cerebellum	Unvaried	Unvaried
	Hypothalamus	Unvaried	Unvaried
	All brain region	Unvaried	Increased
Exogenous hyperthermia	Cerebral cortex	Unvaried	Unvaried
	Cerebellum	Increased	Increased
	Hypothalamus	Unvaried	Unvaried
	All brain region	Unvaried	Decreased

*Increased/Decreased: $p<0.05$; Unvaried: $p>0.05$.

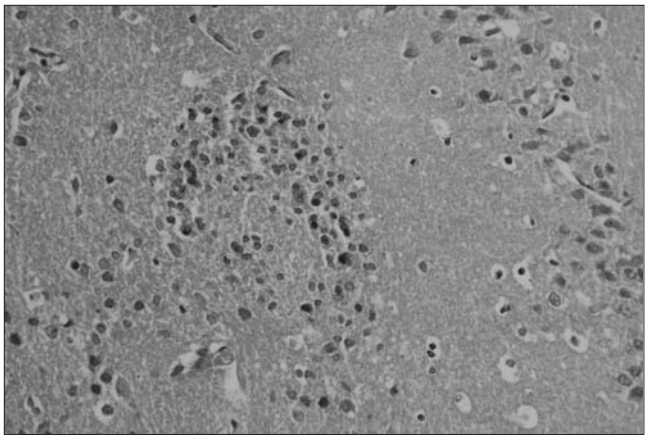

Figure 1. Cerebral cortex sections of a pup from endogenous hyperthermia (41 °C) group showing necrosis (H&E, X40).

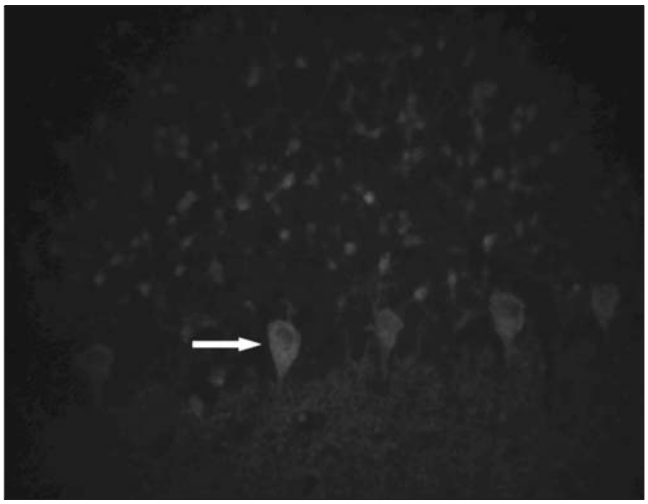

Figure 2. Cerebellum sections of a pup from endogenous hyperthermia (41°C) group showing apoptotic neurons (TUNEL, X100).

Neuronal injury may occur in the hyperthermia phase of febrile process. Hyperthermia can inhibit cell proliferation, increase apoptosis, edema and congestion, disrupts the vascular structure and haemorrhage in perivascular region (9,10). Injury develops at cellular level following hyperthermia and in consequence of this damage brain, edema rises with the increase of blood-brain barrier permeability.

Although there are studies reporting the results based on lipopolysaccharide-induced endogenous hyperthermia (6,8,11), there are limited number of studies investigating the neuronal effects of experimentally-induced exogenous hyperthermia. Our present study uniquely focused on evaluating the [possible] effects of both endogenous and exogenous hyperthermia at 41 °C in rat brain.

In a previously reported study, Heida et al. showed that 50% of rats developed convulsion after fever induced by LPS (6). Onoe and Nishigaki showed that fever caused not only febrile convulsion, but also febrile myoclonus and febrile delirium (12). The association between seizures and hypothalamic neuronal damage was also described (13,14). In

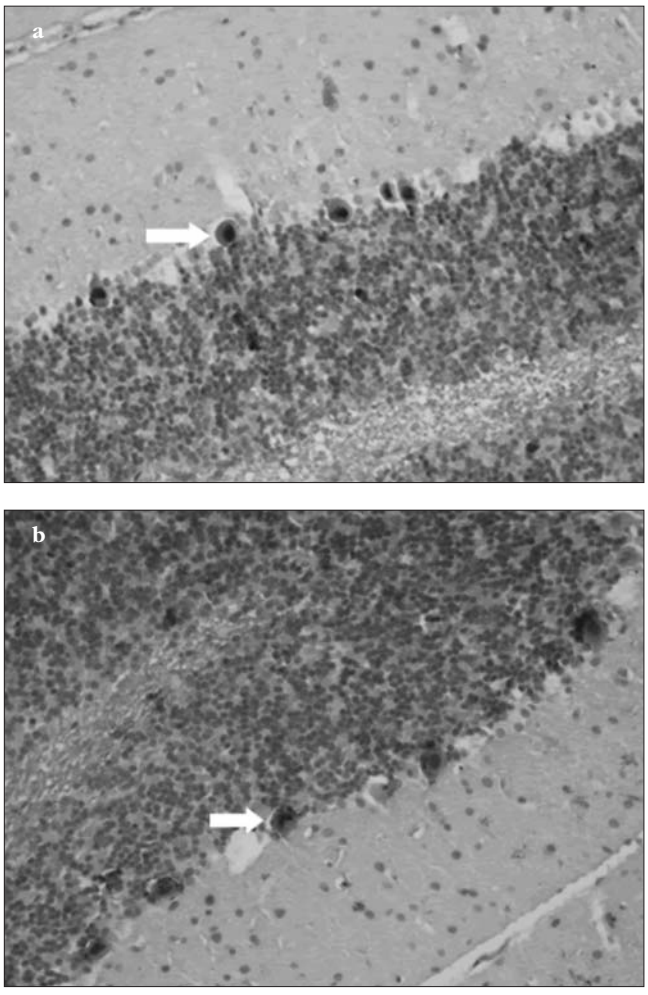

Figure 3. Cerebellum sections of a pup from exogenous hyperthermia (41°C) group showing a- HSP 27 (+) b- HSP 70 (+) cells (Clone, X100).

the present study, endogenous febrile convulsion rate was significantly higher than exogenous febrile convulsion (41%-33%). We can conclude that endogenous, that is LPS induced hyperthermia was associated highly with febrile convulsion and mortality.

In our study, in the LPS administered group, there were significant increases of necrotic cells in cerebral cortex and cerebellum cross sections. While there was a significant decrease of healthy cell count in cerebral cortex, there was a decrease in the healthy cell count in cerebellum and hypothalamus, but it was statistically insignificant. In the

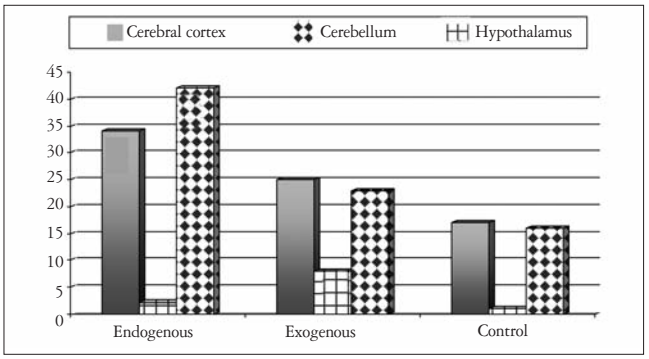

Figure 4. Mean necrotic cell counts in endogenous and exogenous hyperthermia in the different part of brain.

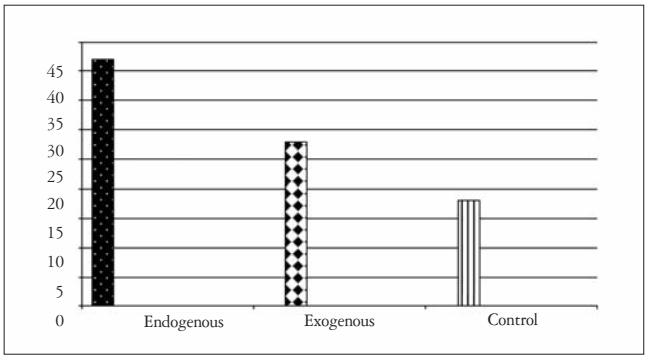

Figure 5. Mean apoptotic cell counts in endogenous, exogenous hyperthermia and control groups in all brain regions together.

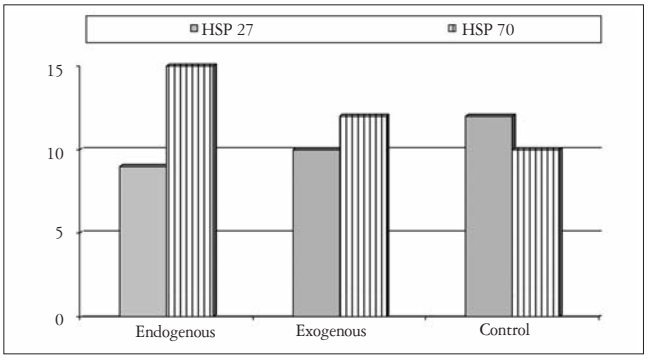

Figure 6. Mean HSP 27 and HSP 70 (+) cell counts in all neuronal regions.

study of McCaughran et al, which had a similar model with our study, they showed that hyperthermia injury of frontal cortex and cerebellum was caused by cholinergic system different from hypothalamus (15). In a case article by Ravid S et al., it has been shown that fever causes necrotizing encephalopathy affecting the thalamus, hypothalamus, brainstem tegmentum, cerebral white matter, and cerebellum (16). Spencer et al. found that after LPS induced fever in rats, brain damage appears especially in hippocampus and amigdala (17). On the other hand, Mouihate and Pittman have shown that fever after LPS injection does not increase cell death in hypocampus, hypothalamus, area postrema, subfornical organ, lamina terminalis and nucleus tractus solitarius (18).

We noticed that one of the basic components of endogenous hyperthermia injury induced by LPS was the significant increase of apoptotic cell number in all neuroanatomic regions. Tomimatsu et al. have indicated that hyperthermia increased the brain damage of immature rats and determined the mechanism of apoptotic cell death (19).

Synthesis of HSP, well known to be overproduced during stress, is increased in cells in which apoptotic processes are induced following hyperthermia (20,21). Heat stroke proteins have a role in cellular regeneration and cellular damage repair (22). Due to stress, particularly the production of HSP70 increased (23-25). We showed that endogenous hyperthermia did not affect HSP 27 and HSP 70 in any brain region examined. The result obtained is different from the ones in some other previous studies (26,27).

Our study also included evaluation of effects of exogenous hyperthermia on neuronal cells. We observed a reduction of healthy neuronal cell numbers of hypothalamus while necrotic cell numbers in cerebellum increased at 41 °C hyperthermia. Similar to the findings of the present study, other researchers such as Ekimova (28) and Khan and Brown (29) also reported that hypothalamus and cerebellum were among the most heat sensitive regions of brain; cerebellar and hypothalamic structures appear to be more heat sensitive compared to cerebral cortex. Sinigaglia-Coimbra et al. reported a heavy damage (78.7%) in hippocampus region of rat brain caused by 38.5°C-40 °C (30).

In our study, expression of both of the heat stroke proteins increased in cerebellum exposed to external hyperthermia. However, we did not observe any significant changes regarding HSP 27 and HSP 70 levels in other parts of rat brains. Belay and Brown reported that level of HSP tend to increase in damaged rat brains by hyperthermia, particularly in Purkinje cells of cerebellum (31). These findings may be an indicator of damage in cerebellum due to hyperthermia and subsequently developing cellular regeneration. It has been proposed that hyperthermia-induced HSP 70 levels may be associated with pre-synaptic and post-synaptic elements. Particularly positioning of HSP 70 at the synapses may facilitate the repair of synaptic proteins which have been damaged by heat stress (26). Presence of HSP 70 in synapses has been proposed to have protective effects.

It has been well documented that hyperthermia inhibits cellular proliferation and increases apoptosis (32-34). In the

present study we observed that 41 °C exogenous hyperthermia caused an increase in apoptotic process in cerebral cortex. Singh demonstrated that cerebral cortex was among the regions where apoptotic changes are observed following the damage from hyperthermia (35). These results are in agreement with our present findings, indicating that apoptotic process may take place in cerebral cortex when temperature increases to 41 °C.

Our study shows that hyperthermia can be harmful for neuronal tissue in brain. Neuronal injury of hyperthermia can be confronted in various ways. Different effects of hyperthermia process on different regions of brain can be explained by the complexity of brain where different neuronal structures and neuronal support structures are present. Heterogenic cellular structures with different neuronal and neurotransmitter contents make it more complex to analyze the effect of fever and hyperthermia as well as the effects of certain drugs on overall process. It has also been well documented that different parts of brain have different metabolisms and neurotransmitter structures (36).

We can conclude that, however, endogenous hyperthermia had more destructive effects in the rat brain regions. On the other hand, in the exogenous hyperthermia group, cerebellar cells were damaged more than they are in cerebral regions.

More comprehensive histochemical trials and clinical results are needed for further findings.

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