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The Effects of Diagnostic Ultrasound Waves on Excitability Threshold and Cellular Apoptosis Induced by Pentylenetetrazole in Hippocampal Neurons

Tanısal Ultrason Dalgalarının, Hipokampal Nöronlarda Pentilentetrazol Tarafından İndüklenen Uyarılabilirlik Eşiği ve Hücresel Apoptoz Üzerine Etkileri

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Abstract

Objective: Ultrasound (US) is a medical imaging technique with various therapeutic and diagnostic applications. This study aimed to investigate the effects of diagnostic US waves with a frequency of 3.5 MHz and intensity of 65 mW/cm^2 on the threshold of neurons' excitability and the apoptosis induced by pentylenetetrazole (PTZ) in rats.

Materials and Methods: Forty-seven-day-old Wistar rats were randomly divided into five groups. The first group was assigned as the control group, the second group was seized by intraperitoneal injection of PTZ, the third, fourth, and fifth groups were given US waves for 5, 10, and 15 minutes, respectively, followed by intraperitoneal PTZ injection. The animals were observed and their behavior, including the seizure duration, the number of seizures, and the seizure cessation time were recorded for 30 minutes. Subsequently, animals' hippocampi were removed in order to measure B-cell lymphoma protein 2 (BCL-2) and BCL-2-associated X (BAX) by using Western blotting techniques.

Results: The results showed that US waves in the diagnostic frequency range with a duration of 5, 10, and 15 minutes significantly increased the seizure duration in the target groups. Furthermore, the simultaneous use of US with the desired times with PTZ increased the number of seizures and prolonged the seizure cessation time. PTZ increased the BAX/BCL-2 proteins ratio, and the concomitant use of US and PTZ intensified the impairment.

Conclusion: This study showed that exposure to US increased the excitability of neurons and exacerbated the seizure effects of PTZ as well as PTZ-induced apoptosis in the rat hippocampal cells.

Keywords: Ultrasound waves, seizures, apoptosis, hippocampus, pentylenetetrazole

Öz

Amaç: Ultrason (US), çeşitli terapötik ve tanısal uygulamalarda kullanılan bir tıbbi görüntüleme tekniğidir. Bu çalışmada, sıçanlarda 3,5 MHz frekans ve 65 mW/cm^2 yoğunluktaki tanısal US dalgalarının nöronların uyarılabilirlik eşiği ve pentilentetrazol (PTZ) tarafından indüklenen apoptoz üzerindeki etkilerinin araştırılması amaçladı.

Gereç ve Yöntem: Kırk yedi günlük Wistar sıçanları rastgele beş gruba ayrıldı. Birinci grup kontrol grubuydu, ikinci gruba intraperitoneal PTZ enjeksiyonu ile nöbet geçirtildi, üçüncü, dördüncü ve beşinci gruplara sırasıyla 5, 10 ve 15 dakika US dalgaları uygulandı ve ardından intraperitoneal PTZ enjeksiyonu ile bu gruplara nöbet geçirtildi. Hayvanlar gözlemlendi ve nöbet süresi, nöbet sayısı ve nöbet bitiş zamanı dahil olmak üzere davranışları 30 dakika boyunca kaydedildi. Daha sonra, Western blot teknikleri ile B-hücresi lenfoma proteini 2 (BCL-2) ve BCL-2-ilişkili X (BAX) düzeylerini ölçmek için hayvanların hipokampusları çıkarıldı.

Bulgular: Sonuçlar, 5, 10 ve 15 dakikalık sürelerle tanısal frekans aralığındaki US dalgalarının hedef gruplarda nöbet süresini önemli ölçüde artırdığını göstermiştir. Ayrıca US'nin istenilen sürelerle eş zamanlı kullanılması PTZ'nin neden olduğu nöbet sayısını artırmıştır ve nöbet bitiş süresini uzatmıştır. PTZ, BAX/BCL-2 protein oranını artırmıştır ve US ve PTZ'nin birlikte kullanımı bozulmayı artırmıştır.

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Sonuç: Bu çalışma, sıçan hipokampal hücrelerinde US'ye maruz kalmanın nöronların uyarılabilirliğini artırdığını ve PTZ'nin nöbet etkilerini ve ayrıca PTZ ile indüklenen apoptozu şiddetlendirdiğini göstermiştir.

Anahtar Kelimeler: Ultrason dalgaları, nöbetler, apoptoz, hipokampus, pentilentetrazol

Introduction

Ultrasound (US) waves have been developed in diagnostic and therapeutic modalities, and their utility in the medical industry is of great value (1,2). US with diagnostic goals is used in neurology, cardiology, obstetrics, and gynecology (3). Monitoring the fetal heart, examining growth rate, estimating fetus age, determining the fetus's position, diagnosing ectopic pregnancy, and examining ovarian and breast tumors are some diagnostic applications of these waves in the field of obstetrics and gynecology (3). Frequencies of 2 to 18 MHz with an intensity up to 720 mW/cm^2 are utilized in diagnostic cases (4). In therapeutic cases, however, the frequency range is about 0.75 to 3 MHz (5), with intensities between 100-10000W/cm^2 (6).

There is some evidence that frequent exposure to US waves has extensive destructive effects on the fetus (7). It has been shown that focused US waves reversibly suppress neural conduction (8). These waves, with their thermal effect, also cause neural apoptosis (9). US radiation causes lower extremity paralysis with myelin damage induced by chromatolysis and axonal degeneration in the spinal cord and peripheral nerves (10,11). It is reported that the myelin membrane is sensitive to US waves which disrupt the development of the nodes of Ranvier and cause alteration of myelination (12). It was also demonstrated that US exposure modulated presynaptic neuron firing rates and increased the dendritic field potential in the hippocampus (13). High-intensity US waves impair neural function, while low-intensity waves repair damaged nerves and improve the rate of transmission and the potential for underlying action in these nerves in mice's tibia (14).

A study by Lee et al. (15) reported that almost no side effect was induced by transcranial US stimulation (TUS) on human participants, and that only one patient suffered from a temporary headache. In another study, in which participants received TUS with the frequency of 0.5 MHz and intensities of 17.12 W/cm2 (I_{SPPA}) and 6.16 W/cm^2 (I_{SPTA}), some ephemeral, low to moderate levels of symptoms were reported, such as muscle twitches, neck pain, sleepiness, itchiness and headache (16).

One of the electrical manifestations of the brain is the occurrence of seizures (17). Seizures are sudden attacks on the brain's electrical activity that can cause loss of consciousness, muscle contractions, repetitive movements, and sometimes sensory disturbances (18). The most common way to cause seizures in laboratory animals is to inject pentylenetetrazol (PTZ), which inhibits gamma-aminobutyric acid (GABA) receptors; thereby stimulating neuronal activities (19), and quickly disturbing the balance between the inhibitory and excitatory systems in the brain (20). In studies, creating a seizure model by PTZ comprises a large number of signaling pathways that cause neuronal impairment, alterations in behavior, intellectual dysfunction, and apoptosis induced by seizure (21).

A few studies have been done regarding the effect of US waves in the diagnostic range on the electrical activity of brain cells, and the effects of US waves on the electrical activity of neurons are not completely understood. Therefore, the current study was conducted to investigate the effects of US waves on the intensified electrical activity of neurons in the brain under the influence of PTZ.

Materials and Methods

Animals and Experimental Design

The current study was performed on 7-day-old male Wistar rats weighing 40-50 grams (provided from animal center, Ilam University of Medical Sciences, Ilam, Iran). The animals were kept in the laboratory with 12-hour light and dark, temperature of 24 ±1 °C for a week in order to acclimatize. The animals were divided into five groups (n=8) as follows:

Control group (sham), PTZ-induced seizure group (PTZ), PTZ+US5, PTZ+US10, and PTZ+US15, groups with 5-, 10- and 15-minutes exposure to US simultaneous with PTZ, respectively. PTZ was purchased from X (CAS 54-95-5). A summary of the experimental design is shown in the Table 1.

Ultrasound Waves Application and Pentylenetetrazole Injections

In the groups with simultaneous US exposing and PTZ injection, first, the US waves with the frequency of 3.5 MHz and intensity of 65 mW/cm^2 were applied for the desired time (US probe was placed on the rat's head). Then intraperitoneal PTZ (60 mg/kg, i.p.) injection was performed (22) in order to induce seizures and observe behaviors.

Seizure Monitoring

Immediately after PTZ injection, seizure symptoms were evaluated and recorded for 30 minutes, and the seizures' durations, the number of seizures, and seizure cessation time were measured. Seizure behavior was monitored according to Racine's standard stages as follows: stage 0: no response; stage 1: facial movements; stage 2: head nodding; stage 3: forelimb clonus; stage 4: rearing and severe forelimb clonus; stage 5: falling back, hind limb extension and, and death (23).

Western Blot Analysis

To measure BAX and B-cell lymphoma protein 2 (BCL-2) in hippocampal tissue by the Western blot method, the animals were anesthetized by ether, and the hippocampi were harvested. Then tissues were promptly placed in the nitrogen tank and stored in an

Table 1. Experimental design

Group names	Ultrasound waves exposure	PTZ injection
Control (sham)	-	-
PTZ (II)	-	*
PTZ+US5 (III)	*(for 5 minutes)	*
PTZ+US10 (IV)	*(for 10 minutes)	*
PTZ+US15 (V)	*(for 15 minutes)	*

*Shows that ultrasound waves/PTZ injection were applied, PTZ: Pentylenetetrazole

-80 °C freezer. Total protein was extracted using the instruction of the kit, and protein concentration was measured by the BCA protein quantification method. Briefly, hippocampal tissues were homogenized under cold conditions. Gel electrophoresis was performed with 10% separation gel and 5% concentration gel. Homogenized hippocampal tissue was exposed to electrophoresis on SDS-PAGE. The isolated proteins were then electro transferred to PVDF membranes and blocked with 5% skim milk and 0.1% tween-20 in tris-buffered saline at room temperature for an hour. The membranes were incubated with the desired primary antibodies then conjugated to the secondary antibody. Chemiluminescence detection of the immune complexes was conducted, and the results were later quantified (Bio-Rad, USA). Protein analysis was accomplished by anti-human ILK (ab-76468, Priab 1/5000, Sec ab1/2000), Bax and Bcl2 (ab- P5498, Priab 1/500, Sigma).

Ethical Issues

The institutional ethical committee of Ilam University of Medical Sciences approved all study protocols (IR.MEDILAM.REC.1395.66) on 2017.03.03.

Statistical Analysis

Statistical analysis was performed by SPSS 23 software. Data were expressed as mean ± standard error of the mean, and One-Way ANOVA, post-hoc LSD, and paired t-test were applied with the significance level of less than 0.05 (p value ≤0.05).

Results

The Effect of PTZ and Concomitant Exposure to Ultrasound Waves on Seizures Duration

As shown in Figure 1, the US exposure time increased seizures duration significantly ($p<0.05$). However, no significant differences were observed between groups of PTZ+US10 and PTZ+US15 ($p<0.05$).

The Effect of PTZ and Concomitant Exposure to Ultrasound Waves on the Number of Seizures

Concomitant use of US waves (with different exposure times) and PTZ, significantly increased the number of seizures compared to the PTZ group ($p<0.05$). It seemed that the US exposure time had an extensive effect on the number of seizures as there were significant differences between the group of PTZ+US15 and the other groups ($p<0.05$; Figure 2).

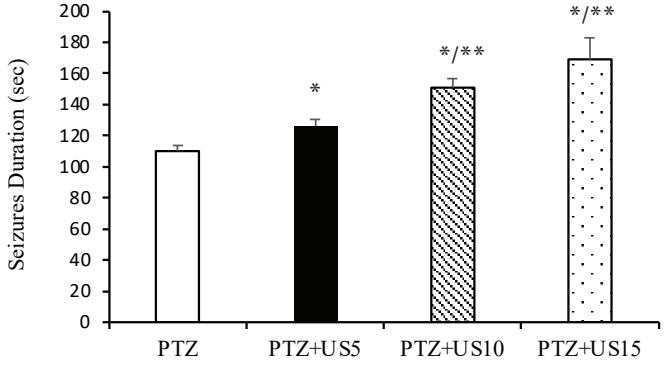

Figure 1. The effect of PTZ and concomitant use of ultrasound on the seizure' durations in four experimental groups; *Shows significant difference compared to PTZ group; **Shows significant difference compared to PTZ+US5 group ($p<0.05$)
PTZ: Pentylenetetrazole

The Effect of PTZ and Concomitant use of Ultrasound on the Seizure End Time

As shown in Figure 3, concomitant use of US and PTZ (with different exposure times) remarkably increased the seizure end time in the target groups compared to PTZ groups ($p<0.05$). However, the US exposure time did not cause any significant differences between the groups of PTZ+US5, PTZ+US10, and PTZ+US15 ($p<0.05$).

Molecular and Cellular Findings

The data showed that the BAX/BCL-2 ratio was considerably elevated in the PTZ group compared to the control group ($p<0.05$). The ratio got higher when US exposure time increased. The ratio was higher in the groups of PTZ+US10 and PTZ+US15 compared to the PTZ group ($p<0.05$; Figure 4).

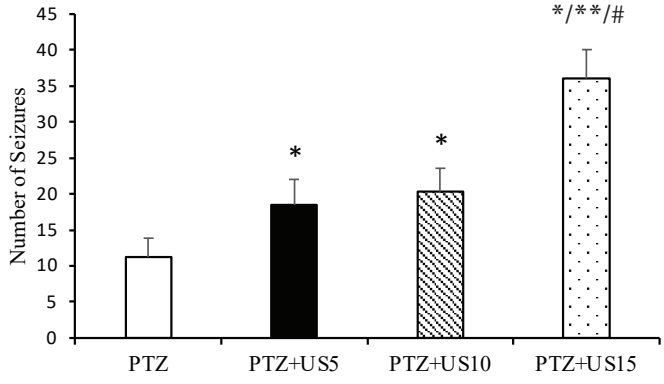

Figure 2. The effect of PTZ and concomitant use of ultrasound on the number of seizures in four experimental groups; *Shows significant difference compared to PTZ group; **Shows significant difference compared to PTZ+US5 group; #Shows significant difference compared to PTZ+US10 group ($p<0.05$)
PTZ: Pentylenetetrazole

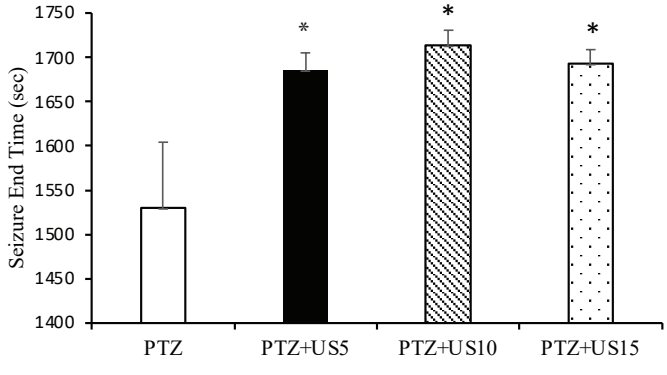

Figure 3. The effect of PTZ and concomitant use of ultrasound on the seizure cessation time in four experimental groups; *Shows significant difference compared to PTZ group ($p<0.05$)
PTZ: Pentylenetetrazole

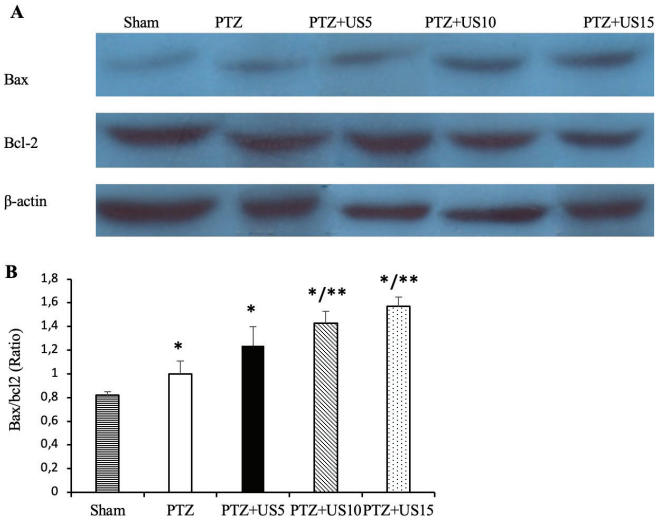

Figure 4. BAX and BCL-2 proteins expression (A) and ratio (B) in five experimental groups; *Shows significant difference compared to sham group ($p<0.05$); **Shows significant difference compared to PTZ group ($p<0.05$)
PTZ: Pentylenetetrazole

Discussion

The main purpose of the present study was to investigate the US effects in the diagnostic range with a frequency of 3.5MHz and intensity of 65 mW/cm^2 on electrophysiological properties of neurons in the PTZ-induced seizures model.

In accordance with the previous studies, PTZ injection caused seizures in the animals (24). PTZ acts by occupying the GABA receptor picrotoxin site and inhibiting the chloride channel (25), then perturbing the balance between excitatory and inhibitory neurotransmitters. As GABA is one of the most critical neurotransmitters in the brain, the concentration of GABA or its receptors in brain tissue reduces following PTZ injection (16,20). Yang et al. (26) found that TUS could also reduce the extracellular level of GABA and raise the extracellular level of dopamine and serotonin.

On the other hand, auto radiographic research on the rat brain reveals that PTZ affects by extending the duration of glutamate binding to *N*-methyl-*D*-aspartate receptors in the dentate gyrus and CA1 areas of the hippocampus (27). It has been shown that intracellular calcium ions are elevated during PTZ-induced seizures. Calcium channels can play a key role in the regulation of seizures by controlling the release of neurotransmitters. PTZ also expedites the neuronal bursting activities by altering the conduction of sodium ion channels, reducing the neuronal excitation threshold, and consequently causing depolarization and seizures (28).

Our study showed that concomitant use of US and PTZ increased the number of seizures, the seizure duration, and seizure cessation time. Although the exact mechanism of US effects on cells is unknown, recent studies have shown physiological effects of these waves in excitable tissues (29,30). Few studies have also evaluated side effects of US exposure in the diagnostic range; however, it has been shown that fetal exposure to US impairs brain function and causes some behavioral problems in rodents (31,32). In humans, focused US causes neonatal weight loss (33), increased left-handedness (34), speech delay (35), and increased heat and pain feeling (36). Fry et al. (37) explained that US waves modulated neurons' activity in the spinal nerves in crayfish, probably due to US effects on membranous lipid, integral protein, and ion channel conductance. Bachtold et al. (13) reported that US with an intensity of 1 W/cm^2 for 5 minutes and a frequency of 3.5 MHz increased the action potential range, while an intensity of 3 W/cm^2 decreased the action potential range. High-frequency US modulates the electrical excitability of neurons which is directly mediated by mechanical forces and not thermal impacts of these waves (38,39). Yoo et al. (40), who investigated both excitatory and inhibitory modulation of TUS in rabbits, found that longer duration of sonication was related with suppressive effects; in comparison, shorter duration was related with excitatory effects. Besides, the higher intensity seems to induce excitatory impacts (41).

Additional studies show that US induces bubble-like bioacoustics cavitation in the intramembranous area between bilayers lipid of the cell's membrane, which causes multiple changes in cells, such as permeability and transitions via cellular mechanic transduction processes and creation of membrane pore form (42). In other words, TUS incites cells by affecting the cell pores and membrane permeability which causes changes that can last for hours (43,44). These changes subsequently boost calcium entry into the cell (44). Many mechanical attributes related to voltage-dependent ion channels and G-protein-coupled receptors can be affected by US waves (45), which open voltage-gated sodium and calcium channels and affect the concentration of excitatory neurotransmitters (40,46). It is recorded that US waves with an intensity of 3 MHz for 40 minutes can increase the concentration of intracellular calcium in fibroblasts; and also affect potassium entry and exit in mouse thymocytes (47). US probably changes the nature of cell membranes and ion channels, possibly by increasing the conductance of calcium or other ion channels and modulates the excitability of neurons (48). There is a variety of evidence related to the role of calcium in homeostasis, control of apoptosis as well as cell death (49). Several reports have shown the effect of different concentrations of calcium on the early and late stages of apoptosis (50), which can induce apoptosis from intracellular sources or increased calcium uptake through membrane channels (49). It has also been reported that blocking calcium receptors reduces BAX levels and caspase-3 activity by preventing intracellular calcium accumulation (51). PTZ shifts calcium from intracellular sources into the cytoplasm, which is ameliorated by calcium channel blockers. PTZ has also been shown to induce apoptosis in cultured hippocampal cells in rats by inducing seizures via caspase-3 and releasing cytochrome C (52,53).

Another mechanism of apoptosis is an increased BAX/BCL-2 ratio. The BCL-2 family includes apoptotic inhibitor and promoter proteins that are key regulators of this process. The BCL-2 protein is a suppressor, while BAX promotes the process of apoptosis (54). Similarly, Skommer et al. (55) reported that seizures caused neuronal apoptosis, especially in the hippocampus, accompanied by increased BAX and decreased BCL-2 proteins. In the present study, in addition to the adverse effect of PTZ, we observed a significant difference between PTZ+US10 and PTZ+US15 groups compared with the PTZ group in terms of the BAX/BCL-2 ratio, which implied an important role of time exposure to US waves.

Study Limitations

The limitation of this study was the low number of animals. A larger number of animals could provide more valid data with less standard error. However, it was impractical due to the policy of Institutional Animal Care and Use Committee of the Medical University of Ilam. Furthermore, our research only assessed US waves with the frequency of 3.5 MHz and intensity of 65 MW/cm^2 (mainly used frequency and intensity in the diagnostic range in studies), and it was inapplicable for us to investigate the other US characteristic levels. However, future studies should consider and compare various frequencies and intensities in the diagnostic spectrum.

Conclusion

In conclusion, the results presented here provide evidence of the deleterious consequences of diagnostic US waves on seizure-like behaviors and of the increased number of seizures, seizure duration, and seizure cessation time in PTZ-induced male Wistar rats. Besides, the simultaneous use of US waves and PTZ causes apoptosis and increases BAX/BCl-2 ratio in hippocampal cells. However, further studies are required to comprehensively elaborate on the mechanism underlying US waves and PTZ in neural cells.

Ethics

Ethics Committee Approval: All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Medical University of Ilam (IR.MEDILAM.REC.1395.66).

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: F.S., Concept: M.R.K., Design: A.M., M.R.K., Data Collection or Processing: N.A., F.S., Analysis or Interpretation: F.S., M.M., Literature Search: M.K., Writing: M.K., M.M., M.R.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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