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# The Synergistic Effects of Ultrasound Waves and Ethanol Exposure on Intracellular Calcium and Reactive Oxygen Species Rates of Hippocampal Cells During the Embryonic Course in Rats

## *Sıçanlarda Embriyonik Süreçte Ultrason Dalgalarının ve Etanol Maruziyetinin Hipokampal Hücrelerin Hücre İçi Kalsiyum ve Reaktif Oksijen Türleri Oranları Üzerindeki Sinerjistik Etkileri*

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## Abstract

**Objective:** Ultrasound (US) is an imaging procedure with various applications, such as checking the fetus during pregnancy. To the best of our knowledge no study have investigated the simultaneous effect of diagnostic US waves and ethanol exposure on intracellular calcium ([$Ca^{2+}$]i) processes and reactive oxygen species (ROS). Thus, in the current study, we first aimed to investigate the impacts of diagnostic US during fetal life on [$Ca^{2+}$]i and ROS in rats' hippocampal cells; then whether the diagnostic US has intensified ethanol effects on these two variables.

**Materials and Methods:** After mating, adult female Wistar rats were divided into six groups as follows: Control group, ethanol group (5 g/kg), groups that were exposed to diagnostic US at frequencies of 3 and 5 MHz for 10 minutes on the 11-13 days of pregnancy, and groups that underwent US waves accompanied with ethanol on the same term of pregnancy. Fourteen days after delivery, the offspring were anesthetized, the hippocampus was isolated, and [$Ca^{2+}$]i was measured using the Flou 3-AM and fluorimetry instrument after cell culture.

**Results:** The results showed that the diagnostic US with 3 and 5 MHz frequencies significantly increased [$Ca^{2+}$]i levels up to 17.8% and 21.8 %, as well as ROS levels by 81.18% and 128.51% compared to the control group. In addition, [$Ca^{2+}$]i levels in the groups with simultaneous exposure to ethanol and US with frequencies 3 and 5 MHz were 19.19% and 23.33% higher than the ethanol group. Besides, the amount of ROS in the groups with simultaneous exposure to ethanol and US with frequencies 3 and 5 MHz were 77.5% and 172.5% higher than the ethanol group, respectively. However, ethanol alone did not alter intracellular [$Ca^{2+}$]i and ROS levels.

**Conclusion:** It is concluded that diagnostic US with 3 and 5 MHz frequencies increase the [$Ca^{2+}$]i and ROS rate, and it also intensifies the effects of ethanol on the [$Ca^{2+}$]i and ROS levels.

**Keywords:** Hippocampus, intracellular calcium, ultrasound, rat, ROS

## Öz

**Amaç:** Ultrason (US), gebelikte fetüsün kontrol edilmesi gibi çeşitli uygulamalarda kullanılan bir görüntüleme işlemidir. Bildiğimiz kadarıyla hiçbir çalışma tanısal US dalgalarının ve etanol maruziyetinin hücre içi kalsiyum ([$Ca^{2+}$]i) süreçleri ve reaktif oksijen türleri (ROS) üzerindeki eşzamanlı etkisini araştırmamıştır. Bu nedenle, bu çalışmada öncelikle fetal yaşam sırasında tanısal US'nin sıçanların hipokampal hücrelerindeki [$Ca^{2+}$]i ve ROS üzerindeki etkilerini; daha sonra da tanısal US'nin bu iki değişken üzerinde etanolün etkilerini artırıp artırmadığını araştırmayı amaçladık.

**Gereç ve Yöntem:** Erişkin dişi Wistar sıçanlar, çiftleşme sonrası; kontrol grubu, etanol grubu (5 g/kg), 11-13. gebelik günlerinde 10 dakika boyunca 3 ve 5 MHz frekanslarında tanısal US uygulanan gruplar ve aynı gebelik döneminde etanol eşliğinde US dalgaları uygulanan gruplar olmak üzere 6 gruba ayrıldı. Doğumdan 14 gün sonra yavrulara anestezi uygulandı, hipokampus izole edildi ve hücre kültüründen sonra Flou 3-AM ve florimetri cihazı kullanılarak [$Ca^{2+}$]i ölçüldü.

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**Bulgular:** Bu çalışmada 3 ve 5 MHz frekanslı tanısal US'nin kontrol grubuna göre [$Ca^{2+}$]i düzeylerini %17,8 ve %21,8; ROS düzeylerini ise %81,18 ve %128,51 oranlarında artırdığını gösterdi. Ayrıca 3 ve 5 MHz frekanslı etanol ve US'ye aynı anda maruz kalan gruplarda [$Ca^{2+}$]i seviyeleri etanol grubuna göre %19,19 ve %23,33 oranlarında daha yüksekti. Ayrıca 3 ve 5 MHz frekanslı etanol ve US'ye aynı anda maruz kalan gruplarda ROS miktarı etanol grubuna göre sırasıyla %77,5 ve %172,5 oranlarında daha yüksekti. Ancak etanol tek başına hücre içi [$Ca^{2+}$]i ve ROS seviyelerini değiştirmedi.

**Sonuç:** 3 ve 5 MHz frekanslı tanısal US'nin [$Ca^{2+}$]i ve ROS seviyelerini artırdığı ve etanolün [$Ca^{2+}$]i ve ROS seviyeleri üzerindeki etkilerini de şiddetlendirdiği sonucuna varıldı.

**Anahtar Kelimeler:** Hipokampus, hücre içi kalsiyum, ultrason, sıçan, ROS

## Introduction

Ultrasound (US) waves are used in diagnostic and therapeutic modalities, with various intensity and frequency ranges (1). Evidence has shown extensive destructive effects on the fetus following frequent exposure to US waves (2), such as speech delay (3), growth restriction (4), body weight, white blood cell count, muscle tone (5), and cellular excitability (1). Negative impacts of short-term US exposure have been shown on mouse blastocysts after being transferred to surrogate mothers, including reduced implantation, increased resorption and stillbirth rates (6). Furthermore, significant decrease in locomotor activity as well as latency in learning capacity and memory were reported in mice exposed to US waves with the frequency of 3.5 MHz and intensity of 65 mW/$cm^2$ (7,8).

The current study investigates whether ethanol and US waves affect calcium ([$Ca^{2+}$]i) and reactive oxygen species (ROS) rates in the hippocampus. It has been well-established that ethanol can be harmful either by its toxic influence and/or involving its metabolites and ROS generation (9). Bailey and Cunningham (10) found that 1 mmol/l ethanol raised ROS generation up to 53%, and 10 mmol/l ethanol prompted ROS generation by 200%. Ethanol consumption has been demonstrated to evoke ROS generation through the induction of the cytochrome P-450 2E1 isozyme6-9 and uncoupling between P-450 2E1 and NADPH cytochrome c reductase (10). We administrated the highest applicable amount of ethanol, 5 mg/kg, to observe its effects on generated ROS of offspring hippocampi. On the other hand, we previously showed that US waves promoted granular cell migration in the cerebellum (11), as well as the excitability of neural cell (1); and one of our suggestions was that these waves had effect on [$Ca^{2+}$]i concentration. US has also increased the permeability of the Chinese hamster ovary (12) and chick embryo 3T3 fibroblast cells to [$Ca^{2+}$]i (13). In *in vitro* conditions, US-induced apoptosis is caused by a boost in [$Ca^{2+}$]i and ROS in myelomonocytic lymphoma U937 cells (14). The biological effects of diagnostic US have been examined on the embryo 6-8 weeks before abortion. Four lysosomal enzyme studies found various degrees of change in malondialdehyde level and superoxide dismutase activity (15). There are still many controversies concerning the relation between ethanol and [$Ca^{2+}$]i. It has been demonstrated that acute exposure to ethanol reduces cytoplasmic [$Ca^{2+}$]i concentration caused by [$Ca^{2+}$]i channel inhibition and [$Ca^{2+}$]i pump activation. In contrast, chronic exposure to ethanol boosts the intracellular [$Ca^{2+}$]i concentration in cells due to activation of passive [$Ca^{2+}$] i transport systems and inhibition of energy-dependent [$Ca^{2+}$]i transport systems (16).

Considering the fact that US waves promote neural cell migration (11) and cellular excitability (1), two probable associative mechanisms are the increase in intracellular [$Ca^{2+}$]i and ROS. Hence, our study firstly investigated the effects of two different frequencies, 3 and 5 MHz with the intensity of 65 mW/$cm^2$ on [$Ca^{2+}$]i in hippocampus cells during the embryonic course and its relation to ROS production; secondly we examined whether ethanol had synergistic effect in regulating these two factors.

## Materials and Methods

### Animals and Experimental Design

Thirty-six adult female Wistar rats of 200-250 g were brought from the animal lab of the Pasteur Institute, Karaj, Iran. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Medical University of Ilam (EC/93/A/115, date: 16.07.2014) and kept in accordance with the Ilam University of Medical Sciences Guidelines. For accommodation and sustainability, the animals were kept for a week in laboratory conditions, 12 hours of darkness and 12 hours of light, and a temperature of 22±2 °C. After mating, the pregnant female rats were randomly divided into six groups (n=6) as follows: Control group (Con), the group in which ethanol (5 g/kg) was injected intraperitoneally on the eleventh day of pregnancy (Etoh), 3 MHz US group, 5 MHz US group, 3 MHz US+ Etoh group, and 5 MHz US+ Etoh group. Fourteen days after delivery, the offsprings were anesthetized using xylazine (10 mg/kg) and ketamine hydrochloride (100 mg/kg) intraperitoneally, hippocampi were cultured, and [$Ca^{2+}$]i and ROS levels were measured.

### Ultrasound Waves Application and Ethanol Injections

In the groups with the simultaneous US exposure and ethanol injection, ethanol (5 g/kg) was injected intraperitoneally, and US waves with the frequencies of 3 MHz or 5MHz and intensity of 65 mW/$cm^2$ were applied in the $11^{th}$ to $13^{th}$ days of pregnancy (Logiq 200 PRO Series, General Electric's, Solingen, Germany).

### Primary Cell Culture

With slight modifications in Fischer's method (1982), primary cultures of hippocampi neurons were acquired from 14-day-old animals (17). The animals' hippocampi were harvested and digested with [$Ca^{2+}$]i /magnesium-free Hank's balanced salt solution containing 0.25% trypsin for 20 minutes at 20 to 25 °C. Through repeated trituration, hippocampus tissues were dissociated, and the cells were seeded at 1×106 cells/cm on poly-l-lysine (10 µg/ml)-coated plates (Gibco). Hippocampus cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 IU/ml penicillin and 100 mg/ml streptomycin in a humidified incubator with 5% $CO_2$ at 37 °C until desired cell density was provided. Hippocampus cells were used between the second and

fifth passages. Cell viability was determined via conventional light microscopy utilizing trypan blue staining. The growth medium consisted of DMEM supplemented with 10% inactivated fetal calf serum, 5.5 mm glucose, and 0.5% (v/v) penicillin/streptomycin.

### Intracellular ROS Measurement

Estimating intracellular ROS levels in hippocampal cells were made utilizing 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA-sigma) (18), Stock solution (1 mM) was given in DMSO, then diluted to 10 µM. The cells were washed twice and later hatched with the H2DCFDA solution for 30 minutes in the dark (37 °C incubators in a 5% $CO_2$). DCF Fluorescence was then determined at 485 nm excitation and 528 nm emission wave lengths utilizing a microplate reader fluorimetry (FLx800, Bio-Tek, USA).

### [$Ca^{2+}$]i measurement

[$Ca^{2+}$]i of hippocampal cells was dictated by [$Ca^{2+}$]i fluorescent prob flu3-AM (Sigma-Aldrich) (Aton et al., 2006). Shortly, aliquots of 1 ml hippocampal cells suspensions (1×106 cells/ml) were washed with buffer A (Phenol red free DMEM containing 10-mM HEPES (4-(2-hydroxyethyl) piperazine- 1-ethanesulfonic acid, pH7.0) and re-suspended in 200 µl of buffer A. Afterwards, 0.4 µl of Fluo 3-AM (1.0M in DMSO) was added. The cells were incubated at room temperature for 30 minutes and washed with buffer B (DMEM containing 10 mM HEPES, 5% fetal calf serum, and pH 7.4) prior to measure. Flow cytometric analysis of hippocampal cells [$Ca^{2+}$]i was carried out using a FACscan caliber™ flow cytometer (Becton-Dickinson, San Jose, CA, USA) (19).

### Statistical Analysis

Statistical analysis was conducted by SPSS 23 software, and data were expressed as mean ± standard error of mean. One-Way ANOVA and Dunnett's post-hoc test were applied to compare [$Ca^{2+}$]i differences among six groups with a significance level of less than 0.01 (p value ≤0.01).

## Results

### The Effects of Ethanol and Ultrasound Waves on [$Ca^{2+}$]i

Diagnostic US caused a significant increase in [$Ca^{2+}$]i of the hippocampus cells. The increase rates were 17.08% and 21.8% due to frequencies of 3 and 5 MHz, respectively, compared to the Con ($p<0.001$), illustrating a frequency-dependent manner (Figure 1).

Ethanol alone caused no significant change in [$Ca^{2+}$]i of hippocampus cells; however, concurrent use of ethanol in combination with US increased such effects. The amounts of [$Ca^{2+}$]i in 3 MHz + Etoh and 5 MHz + Etoh groups were 19.19% and 23.33% higher than Etoh group, respectively ($p<0.05$) (Figure 1).

### Effects of Ultrasound and Ethanol on ROS

The results demonstrated the cumulative effects of US on ROS. US with the 3 MHz and 5 MHz frequencies caused 81.18% and 128.51% increases in the level of ROS, respectively, compared to the Con ($p<0.001$), illustrating a frequency-dependent manner (Figure 2).

Ethanol alone caused no significant change in intracellular ROS levels; however, concurrent use of ethanol in combination with US increased such effects. The amounts of ROS in 3 MHz + Etoh and 5 MHz + Etoh groups were 77.5% and 172.5% higher than Etoh group, respectively ($p<0.05$) (Figure 2).

### Comparison of changes in [$Ca^{2+}$]i and ROS Due to Ethanol and Ultrasound Effects

The data showed no significant effect of ethanol on [$Ca^{2+}$]i and ROS; however, US with the 3 MHz and 5 MHz frequencies caused a significant increase in [$Ca^{2+}$]i and ROS. It was noted that the elevation of ROS was significantly higher than that of [$Ca^{2+}$]i ($p<0.001$) (Figure 3).

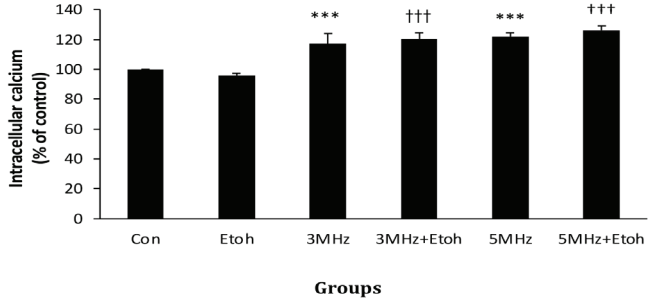


**Figure 1.** Comparison of [$Ca^{2+}$]i among Con, Etoh, 3 MHz, 3 MHz + Etoh, 5 MHz, and 5 MHz +Etoh groups (n=6)
****Significant differences between 3 MHz and 5 MHz groups vs. Con group, $p<0.001$, †††Significant differences between 3 MHz + Etoh and 5 MHz + Etoh groups vs. Etoh group, $p<0.001$.*

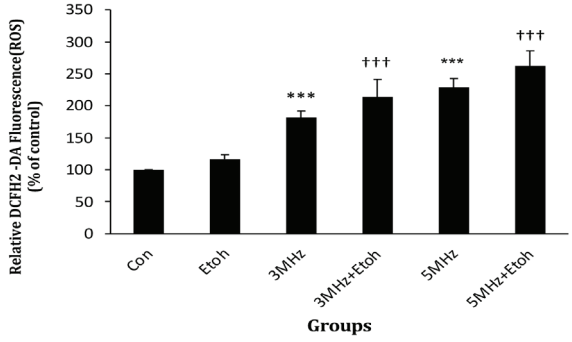


**Figure 2.** Comparison of percent average of reactive oxygen species among Con, Etoh, 3 MHz, 3 MHz + Etoh, 5 MHz, and 5 MHz + Etoh groups (n=6)
****Significant differences between 3 MHz and 5 MHz groups vs. Con group, $p<0.001$, †††Significant differences between 3 MHz + Etoh and 5 MHz + Etoh groups vs. Etoh group, $p<0.001$.*

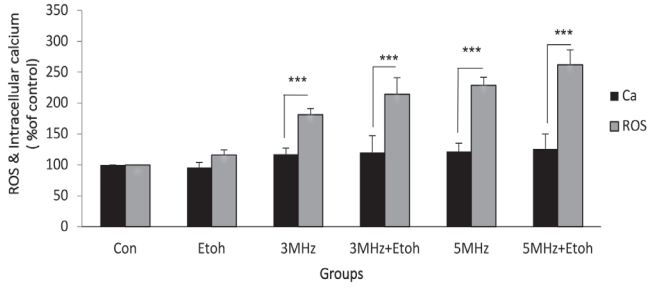


**Figure 3.** Comparison of [$Ca^{2+}$]i and reactive oxygen species within groups of Con, Etoh, 3 MHz, 3 MHz + Etoh, 5 MHz, and 5 MHz + Etoh groups (n=6)
****Significant differences withing each group, $p<0.001$*

## Discussion

The main purpose of current research was to investigate the impacts of US waves with two different frequencies, 3 MHz and 5 MHz and the intensity of 65 mW/$cm^2$ (widely used for diagnostic purposes in human) on [$Ca^{2+}$]i in rats hippocampus neural cells and its connection to ROS production during the embryonic course. In mice, gestational days from 10 to 16 are vital for development of various regions of the body (20), including the brain cells' rapid proliferation and migration to the outer layer of cortex (21). This phenomenon justifies the higher sensitiveness of the mice brain to US-induced behavioral changes in the early fetal term (22). We selected days $11^{th}$ to $13^{th}$ of gestation to investigate the effect of diagnostic US on hippocampal [$Ca^{2+}$]i. Other studies have also shown the effects of US but in the therapeutic range on [$Ca^{2+}$]i in various cells. Mummery (23) revealed that US with the frequencies of 1.5 and 3 MHz and the intensity of 2 W/$cm^2$ induced [$Ca^{2+}$]i uptake growth via 3T3 fibroblasts in an *in vitro* study (13). They reported that this wave increased the [$Ca^{2+}$]i influx without damage to the cell membrane. It has also been found that US stimulation in primary rodent chondrocytes has increased the intracellular augmentation of [$Ca^{2+}$]i (24). In the current study, an elevation in [$Ca^{2+}$]i depended on the frequency of the emitted US waves, as waves with frequencies of 3 and 5 MHz increased [$Ca^{2+}$]i respectively by 17.08% and 21.8%.

Recently, Rosenblatt et al. (25) demonstrated that the pulsed-focused US caused cytosolic [$Ca^{2+}$]i elevation and ROS formation leading to DNA deterioration in tumor cells. In addition, US can overcome the blood-brain barrier, an enormous obstacle to nanoparticle/drug penetration into the brain, demonstrating its affective mechanical role in the brain (26). Investigation on the Chinese hamster ovary cells showed that US caused [$Ca^{2+}$]i increase in the exposure of albumin-encapsulated Optison microbubbles (27). Deng and colleagues reported that US increased the transmembrane current of Xenopus oocyte due to reduced membrane resistance and pore formation (28). Recently, it was shown that focused US with activation of [$Ca^{2+}$]i-selective mechanosensitive ion channel induced the incremental calcification by [$Ca^{2+}$]i- and voltage-gated channels as well as provoking a burst firing signals. It is established that inhibition of these ion channels causes decreased responses to US, whereas over-expressing them drives stronger US stimulation (29).

Increased [$Ca^{2+}$]i may be due to the generated heat US waves on the cell membrane. Tarantal et al. (5) documented that an increase of 0.6 °C in temperature resulting from diagnostic US exposure in macaques caused an upsurge in the transport of ions across the membranes (30). In addition to the probable thermal effect of diagnostic US on increasing membrane permeability, the elevation in ROS production could also enhance this permeability. Increasing the transfer of [$Ca^{2+}$]i from the cell membrane, endoplasmic reticulum, and the mitochondria inside cells can be caused by destructive ROS (31). Honda et al. (14) examined the effect of continuous 1 MHz US at the intensity of 4.9 W/$cm^2$ on human myelomonocytic lymphoma U937 cells and reported changes of [$Ca^{2+}$]i ion concentration in individual cells involving ROS generated from mitochondria in the regulation of apoptosis (14). It is reported that US with the frequency of 1 MHz induces a considerable influx of [$Ca^{2+}$]i over 3 to 4 seconds in Chinese hamster ovary cells. Furthermore, as an $H_2O_2$ scavenger, catalase reduced the [$Ca^{2+}$]i influx, implying that ROS is also effective in membrane permeabilization (12). However, in this study, the total [$Ca^{2+}$]i in mitochondria, endoplasmic reticulum and cytoplasm [$Ca^{2+}$]i, was measured, and the [$Ca^{2+}$]i accumulation is likely because of increased membrane permeability to this ion.

In the present study, ethanol consumption did not cause any changes in built-up of [$Ca^{2+}$]i. The effect of ethanol on altering [$Ca^{2+}$]i disposition has been of great interest to researchers in several investigations. Still, several investigations on free [$Ca^{2+}$]i levels have revealed that ethanol causes [$Ca^{2+}$]i release from internal stores in *in vitro* situations (32). Studies utilizing synaptosomes have also reported that acute exposure to ethanol drives to the elevation of [$Ca^{2+}$]i resting levels (33). However, high ethanol concentrations were relatively required to produce such effects, and chronic ethanol exposure had no impact on synaptosomal [$Ca^{2+}$]i (34). During acute ethanol exposure, the growth in resting synaptosomal [$Ca^{2+}$]i is assumed to be the consequence of the [$Ca^{2+}$]i release from the endoplasmic reticulum in preference to the other mechanisms, namely inositol trisphosphate (IP3) (35). On the other hand, it has been shown that [$Ca^{2+}$]i influx through the voltage- and NMDA-operated [$Ca^{2+}$]i channels is susceptible to ethanol inhibition in newborn rats' neural cells (32). Ethanol interferes voltage-gated [$Ca^{2+}$]i channels in several preparations, such as identified neurons of Aplysia (36) and rat hippocampus slices (37). In the present study, ethanol did not cause changes in [$Ca^{2+}$]i; however, concurrent use of ethanol with US strengthened the effect of US on the elevation of [$Ca^{2+}$]i.

Many extrinsic or intrinsic mechanisms seem to cause a boost in intracellular ROS, such as altering the intracellular antioxidant level, increasing surrounding oxygen deposition, exposing cells to hydrogen peroxide, or expressing specific oncogenes (38). Free radicals can be formed due to the X- and gamma rays, ultraviolet and microwave radiations, inflammation, alcohol intake, and specific drugs (39,40,41,42,43). ROS can impair cell structures, nucleic acids, lipids, and proteins at toxic levels (44). The present study demonstrated a boost in ROS levels due to diagnostic US in a frequency-dependent manner. Such an increase in the frequency of 5 MHz was 48% more than that of the 3 MHz frequency. In accordance with our study, it has been shown that low-intensity continuous US for 5 minutes remarkably enhances ROS generation in articular cartilage (45). One factor that enhances ROS production is the elevation of [$Ca^{2+}$]i (14,31,46). In a study on the human leukemic cells, [$Ca^{2+}$]i oscillations accompanied by radical hydroxyl production have been reported due to US waves with the frequency of 1.1 MHz and the intensity of 1 and 2.1 W/$cm^2$ (47). Evidence suggests that [$Ca^{2+}$]i accumulation results from intracellular store deficit or the influx of extracellular medium [$Ca^{2+}$]i is a sign that foregoes apoptosis (31). [$Ca^{2+}$]i increases and activates [$Ca^{2+}$]i-dependent enzymes, including phospholipase A2, xanthine oxidase, and nitric oxide synthase produce free radicals (48,49). In the present study, we showed that increases in [$Ca^{2+}$]i and ROS production in a frequency-dependent manner were correlated. Taken together with the shreds of evidence showing the role of [$Ca^{2+}$]i in the cell death in both *in vitro* and *in vivo* conditions, our study suggests that diagnostic US waves cause an increase in [$Ca^{2+}$]i and ROS production, facilitate the hippocampus cell death, and consequently compel learning disorders in animals (50,51). It is suggested to investigate such effects with frequencies and intensities applied in diagnostic techniques on cell apoptosis in different brain regions.

### Study Limitations

One limitation of this study was the difficulty in accessing the hippocampi of the offspring's brain. Another was the number of female animals. That is to say, a larger number of animals could provide more valid data with less standard error. However, it was impractical due to the policy of the Institutional Animal Care and Use Committee of the Medical University of Ilam. Lastly, each rat's offspring numbers varied, making the data analysis more challenging.

## Conclusion

Our study demonstrated an increasing effect of diagnostic US on [$Ca^{2+}$]i and ROS in hippocampal cells. We also found that ethanol alone could not alter the mentioned parameters; however, concurrent use with US could intensify the cumulative effect of these waves.

### Ethics

**Ethics Committee Approval:** All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Medical University of Ilam (EC/93/A/115, date: 16.07.2014).

**Informed Consent:** Not necessary.

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

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

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

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