Original Article

Damage to Hippocampus of Rats after Being Exposed to Infrasound

ZHANG Meng Yao[1,^], CHEN Chen[2,^], XIE Xue Jun[3], XU Sheng Long[3], GUO Guo Zhen[3,#], and WANG Jin[3,#]

1. Beijing Institute of Biotechnology, Fengtai District, Beijing 100071, China; 2. Department of Blood Transfusion, Xijing Hospital of Fourth Military Medical University, Xi'an 710032, Shaanxi, China; 3. Department of Radiation Medicine, Faculty of Preventive Medicine, Fourth Military Medical University, Xi'an 710032, Shaanxi, China

Abstract

Objective The objective was to observe damage of hippocampus in rats after exposure to infrasound, and to assess HSP70 expression in hippocampus.

Methods SD rats in the experimental group were exposed to 140 dB (8 Hz) infrasound for 2 h per day for 3 days. The morphology of the hippocampus was examined by transmission electronic microscopic (TEM). Cell apoptosis was observed by TUNEL staining at 0 h, 24 h, 48 h, and 2 w after exposure. HSP70 expression was detected by immunohistochemistry (IHC) and Western blotting (WB).

Results TEM showed that hippocampus was significantly damaged by exposure, and exhibited recovery 1 week after exposure. The TUNEL data showed that neuronal apoptosis after exposure was significantly higher than in the control rats at 24 h and 48 h, and the apoptotic cells decreased one week after exposure. IHC and WB showed HSP70 expression was significantly higher in the exposed rats, peaked at 24 h.

Conclusion Exposure to 140 dB (8 Hz) infrasound for 2 h per day for 3 days appeared to induce damage to the hippocampus of rats, based on changes in ultrastructure and increased cell apoptosis. However, recovery from the damage occurred overtime. HSP70 expression also increased after the exposure and decreased by 48 h.

Key words: Infrasound; Hippocampus; Apoptosis; HSP70

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INTRODUCTION

Infrasound is acoustic energy with frequencies up to 20 Hertz, which is extensively emitted into the surrounding environment by industrial manufacturing, natural events, and even the human body[1-2]. Currently, people are paying increasing attention to infrasound as one of the most important contributors to environmental pollution[3]. Each part of the human body always vibrates in a specific rhythmic, with a frequency that is between 2 and 16 Hz, which is almost the exact range of infrasound. Therefore, the human may be damaged by infrasound.

Previous research showed that the brain is one of the most sensitive target organs of infrasound[4]. Infrasound of specific parameters may cause changes in learning and memory function, as well as

^ZHANG Meng Yao and CHEN Chen contributed equally to the work.

#Correspondence should be addressed to WANG Jin, Tel: 86-29-84773401, Fax: 86-29-84774873, E-mail: wangjinn@fmmu.edu.cn; GUO Guo Zhen, Tel: 86-29-84773407.

Biographical notes of the first authors: ZHANG Meng Yao, female, born in 1988, PhD, assistant research fellow; CHEN Chen, female, born in 1974, master, associate chief technologist.

the structure of the brain[5-6]. Research also demonstrated that the apoptosis of hippocampal cells was closely related to learning and memory disabilities[7]. However, most previous studies have focused on biological changes caused by continuous electromagnetic waves which may last for weeks even months[8-9]. During preliminary testing, we observed that rats exposed to infrasound at 140 dB (8 Hz) for 2 h per day for 3 days exhibited longer escape latencies and less time staying in quadrant *P* of the Morris Water Maze (MWM). In this study, we applied infrasound at 140 dB (8 Hz) for 2 h per day for 3 days to study morphological changes in the hippocampus and the apoptosis of hippocampus cells for the first time, and the mechanism through which learning and memory disabilities are caused by infrasound.

The heat shock proteins (HSPs) are a family of conserved and ubiquitously expressed proteins. The HSP70s are important for main taining the body's steady state, and help to protect cells from stress[10-11]. HSP70 proteins also protect cells from thermal and oxidative stress and other stresses. These stresses normally lead to protein damage, for instance, partial unfolding, and even aggregation. HSP70s prevent partially denatured proteins from aggregating, and allow them to refold by temporarily binding to hydrophobic residues produced by stress[12]. HSP70s also seem to participate in the disposal of damaged or defective proteins. Finally, HSP70s directly inhibit apoptosis by blocking the recruitment of the key proteins in the apoptosis pathway[13].

The expression of HSP70 in the central nervous system is a sign of neuronal excitation. Normally, brain tissue expresses only a small amount of HSP70[14]. However, when tissue is damaged, the proteins secreted by the damaged cells will induce an increased expression of HSP70, which can protect neurons[15]. Hence, the study of the expression of HSP70 in the hippocampus of rats damaged by exposure to infrasound, may lead to potential treatments for diseases caused by infrasound.

MATERIALS AND METHODS

Infrasound Exposure Apparatus

The experimental infrasound system consisted of an air compressor, an air modulator, an infrasound tank, and a monitoring platform. The infrasound tank was equipped with alight, temperature sensor, humidity sensor, infrasound microphones, and two cameras. The acoustic signal and the responses of the rats were detected by the infrasound microphones and cameras. The monitoring platform controlled the infrasound frequency and analyzed the acoustic signals received by the microphone, and modulated the sound intensity and the spectral characteristics. The experimental infrasound frequency was 8 Hz, the sound pressure level was 140 dB, and exposure time was 2 h once a day for 3 days in the study.

Animals and Experimental Groups

The experimental protocol used in this study was approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University and the study was conducted according to the Guidelines for Animal Experimentation of the Fourth Military Medical University (Xi'an, Shaanxi, China). Male Sprague-Dawley (SD) rats weighing 100-110 g were obtained from the Animal Center of the Fourth Military Medical University (Xi'an, China). The animals were housed in stainless-steel cages in a temperature-controlled room, with a 12/12 light/dark cycle, and were allowed free access to semi-purified rat chow and pre-prepared drinking water.

Healthy male SD rats were divided into a sham group and an exposed group, which was exposed to 140 dB (8 Hz) infrasound for 2 h per day for 3 days. Before exposure, the rats were adapted to the environment of the infrasound tank. The observation points were 0 h, 24 h, 48 h, 72 h, 1 w, 2 w, and 4 w after exposure to the infrasound. Each observation points contained included 18 rats, 6 for HSP-70 immunohistochemistry and TUNEL staining, 6 for HSP-70 Western Blotting, and 6 for the TEM protocol.

TEM Examination of the Hippocampus of the Rats

A transmission electronic microscopic (TEM) examination was conducted at the Center Laboratory of the Fourth Military Medical University, using a JEM-100SX electronic microscope (Hitachi, Tokyo, Japan). At 0 h, 24 h, 48 h, 72 h, 1 w, 2 w, and 4 w after infrasound exposure, 6 animals from the sham group or the exposed group were anaesthetized with 60 mg/kg sodium pentobarbital, IP. The heart was exposed and the left ventricle was perfused with 0.9% saline, followed by perfusion for 2 h with a fixative consisting of one part 4% lanthanum nitrate and two parts 6% glutaraldehyde in 0.1 mol/L sodium cacodylate (pH 7.40-7.50). At the end of the brain perfusion, the hippocampus was

isolated and cut into 1 mm^3 pieces. The isolated tissues were immersed in 4% glutaraldehyde for 2 h, and then washed twice with PBS. The tissues were immersed in 1% osmium tetroxide for 2 h, and then washed again with PBS for 5 min. After being embedded in resin, the specimens were heated at 60 °C for 48 h. The sections were dyed with acetic acid uranium and lead, mounted on copper grids (200 meshes), and examined under TEM.

In Situ TUNEL Assay

The method of TdT-mediated dUTP-biotin nick end labeling (TUNEL) was used to detect cell apoptosis at 0 h, 24 h, 48 h, 2 w after exposure. We used TUNEL detecting kits (TBD2020POD, Haoyang Biological product technology, Tianjin, China) for in situ end labeling of DNA breaks in nuclei. The sections were dewaxed in xylene and dehydrated through graded alcohol concentration and washed in PBS (pH 7.4). Endogenous peroxidase was inactivated by covering the slides with 0.3% H_2O_2-PBS for 5 min, then rinsing the slides with a balancing solution for 10 min. A reaction buffer containing TdT enzyme and digoxin-dUTP was added to cover the slides, and then they were incubated in a humid atmosphere at 37 °C for 1 h. Immersing the slides in a wash solution for 15 min terminated the reaction. After blocking with 2% BSA for 10 min, the slides were covered with anti-digoxin-peroxidase complex and incubated for 30 min at 37 °C, washed in PBS, and stained with diaminobenzidinete trahydrochloride (DAB). The slides were stained with a hematoxylin counter-stain. The control reaction consisted of omission of TdT, and the substitution of PBS.

HSP-70 Immunohis to Chemistry

The animals were anaesthetized with 60 mg/kg, IP of sodium pentobarbital, and then perfused transcardially with 100 mL of saline followed by 250 mL of 4% formaldehyde in 0.01 mol/L phosphate buffer at pH 7.4. After perfusion, the hippocampus was removed and post-fixed for 24 h in the same solution, and then embedded in paraffin. Coronal sections of 3 μm were consecutively cut, and one section from every five sections was chosen for immunohistochemistry.

The sections were dewaxed in xylene and dehydrated through graded alcohol concentration and washed in PBS (pH 7.4). The sections were incubated with 3% hydrogen peroxide in methanol for 15 min, to block endogenous peroxidase activity and then immersed in a citrate buffer (pH 6.0) at 95 °C for 10 min. After being was hed three times in PBS, the sections were incubated for 2 h at room temperature with polyclonal antibodies specific to HSP70 (dilution 1:150). Following the washing in PBS, the sections were sequentially incubated with biotinylated link secondary antibody and peroxidase labelled streptavidin for 1 h. The sections were again washed three times in PBS and incubated in the chromogen (DAB) substrate, followed by counterstaining in hematoxylin. The sections were mounted in DPX mountant. Parallel control slides were run with all batches including positive and negative controls. The integrated optical density (IOD) of positive cells in sections was analyzed by Image-pro plus 6.0 software (USA). All sections were then evaluated by an experienced pathologist.

HSP70 Western Blotting

Protein samples of rat hippocampus, which were extracted by a five-fold volume of ice-cold lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium desoxycholate, 1% EDTA-2Na and 2% (v/v) protease inhibitor cocktail) were used for determining the protein level of HSP70 at 0 h, 24 h, 48 h, 72 h, 1 w, 2 w, 4 w, after exposure. Samples (30 μg) were separated in a 10% Tris-HCl precast gel for polyacrylamide electrophoresis at 120 V for 90 min, then transfered to polyvinylidene fluoride membranes with 55 V at 4 °C for 120 min. After that, the membranes were blocked in TBS, containing 5% nonfat milk and 0.5% Tween-20, for 60 min at room temperature, and then were incubated overnight at 4 °C with primary antibodies against HSP70 (dilution 1:200). The membranes were washed with TBS containing 0.5% Tween-20 before incubation with the secondary antibodies at a 1:5000 dilution for 1 h at room temperature, and then the membranes were developed using enhanced chemiluminescence-detecting reagents (Western Lightening Kit Millipore, USA). The intensity of each target band was semi-quantified using the Quantity One imaging software (Bio-Rad Laboratories, Hercules, CA, USA). Data were analyzed by the ratio of densitometric intensity of target bands and GAPDH bands in the same membrane.

Statistical Analysis

Western Blot data were analyzed by ANOVA, followed by post-hoc, pairwise comparisons, and the Dunnett-*t* test. The histopathologic evaluation data

of HSP70 and TUNEL staining were analyzed using the Kruskal-Wallis H test; multiple-comparisons were made using the Nemenyi test. Values of *P* less than 0.05 were considered to be statistically significant.

RESULTS

TEM

The hippocampal neurons in the control group had a complete structure and normal cell organelles (Figure 1A). For example, the nucleus was clear, the nucleoli was obvious, the rough endoplasmic reticulum was abundant, the mitochondria were orderly, the mitochondrial crista was clear, and the synaptic structure was normal. The capillary endothelial cells and the blood brain barrier had structural integrity.

The hippocampal tissue was broken in the 0 h group, and the space of capillaries around it was increased significantly; neuron cell edema and mild swelling of the mitochondria were observed, and the mitochondrial cristae were unclear. There were several microglia cells infiltrating the periphery of the neurons (Figure 1B). In the 24 h group, edema was observed in area the surrounding the capillaries, and platelet aggregation in was seen some capillaries' cavities. The increasing electron density mitochondrial swelling and endoplasmic reticulum expanding were observed in some of neurons. The nuclear chromatin was condensed to the edge of nuclear membrane, which was similar to the early stage of cell apoptosis (Figure 1C). The nerve cells in the 48 h group basically had a normal morphology, but there also were some abnormal cell organelles. For example, the endoplasmic reticulum had a mild expansion, and the mitochondrial cristae had a slight expansion too. In addition, the myelinated nerve fibers myelin degeneration were observed (Figure 1D). The hippocampal cells in the 72 h group had a morphology similar to the 24 h group, such as a mild expansion of the endoplasmic reticulum expansion and mitochondrial cristae, and the infiltration of a few glial cells (Figure 1E).

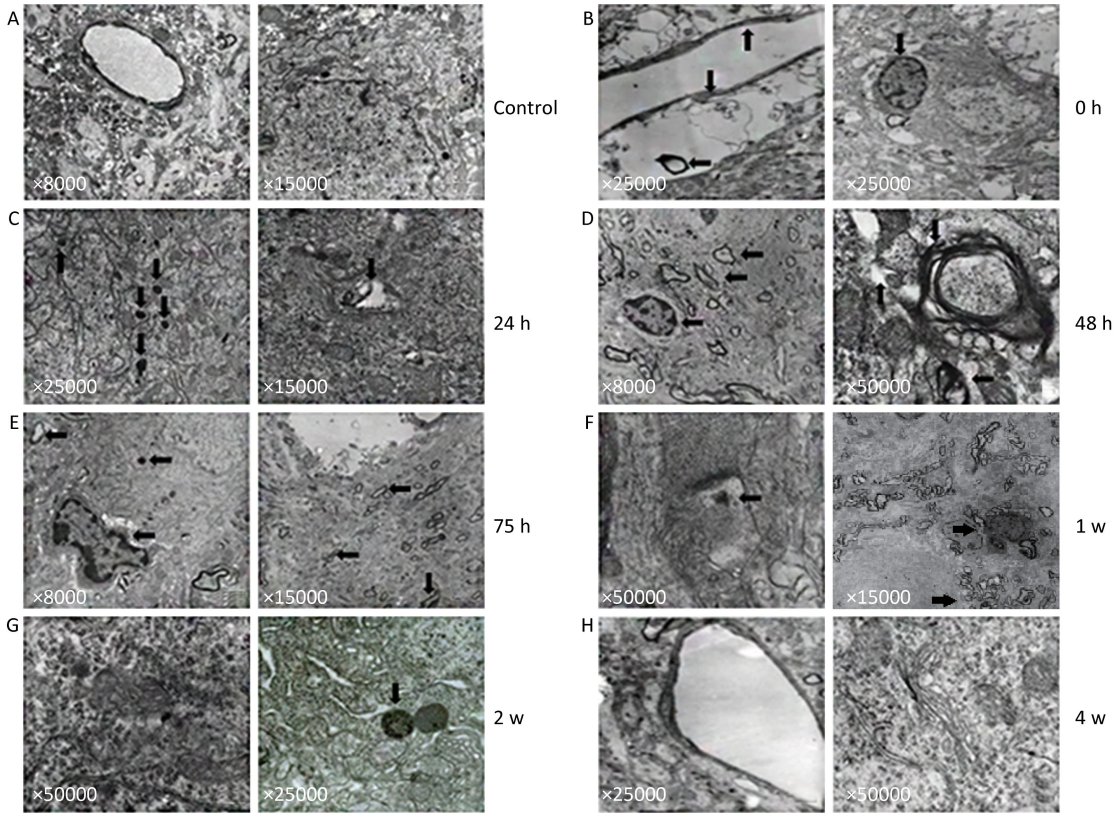

Figure 1. The TEM results. The arrows showed the damaged part.

In the 1 w group, cell lysosome-like structures increased. There was infiltration of a few glial cells. Synaptic structural showed abnormality (Figure 1F). In the 2 w group, the endoplasmic reticulum mildly expanded, mitochondrial cristaes lightly expanded, and showed a few microglias infiltration. There was no edema in the space around the capillaries (Figure 1G). In the 4 w group, the hippocampal cells had a normal structure and clear nucleoli, and the euchromatin was mainly condensed, with no edema of the capillaries (Figure 1H).

TUNEL

Apoptotic cells were not seen in the sham group. The neuronal apoptosis in the hippocampus was significantly increased in the exposure group at 24 h and 48 h (Figure 2). The data also showed that the percentage of apoptotic cells decreased one week after exposure.

HSP-70 Immunohistochemistry

Cells with positive HSP70 immunostaining were not found in the sham group. HSP70 immunostaining cells were detected in the exposure groups (0 h, 24 h, 48 h, 72 h, 1 w, 2 w, and 4 w) after infrasound exposure, especially in the 24 h group (Figure 3).

HSP-70 Western Blotting

Western Blotting results showed that the protein level of HSP70 was significantly higher in the exposure groups compared with the sham group, especially the 24 h exposure group. These results were similar with those for the immunehisto-chemistry (Figure 4).

DISCUSSION

The potential health risks from exposure to infrasound recently have become a public issue[16]. Therefore, it is important to study the biological effects of infrasound that could lead to tissue damage. Previous studies have shown that cognitive functions can be strongly impaired by infrasound. The results showed that rats exposed to infrasound of 16 Hz at 140 dB for 14 days exhibited longer escape latencies after day 2 and less time in quadrant *P* of the Morris Water Maze (MWM)[17]. It seems that exposure of 130 dB caused more severe effects than that of 90 dB. They also showed cortical and hippocampal damage after exposure to infrasound at 90 as well as 140 dB. Most former studies have focused on the biological effects of continuous electromagnetic waves, whereas few studies have examined exposure to higher intensity infrasound for shorter durations. Our experiment further advances under standing about the damages of infrasound on humans and animals, using a limited daily exposure to infrasound over 3 days.

The TEM analysis revealed that the morphological

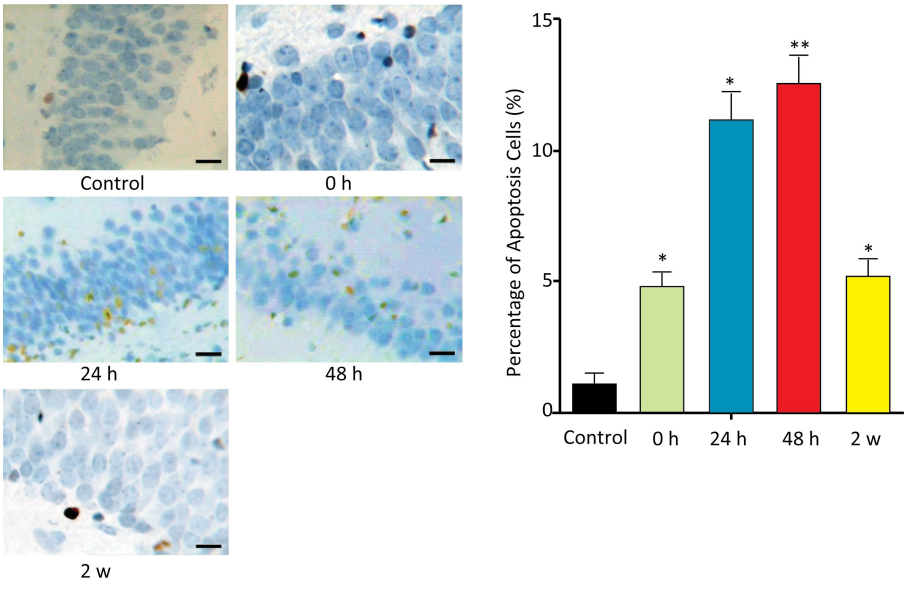

Figure 2. The TUNEL results. The histogram shows the percentage of apoptotic cells calculated from twenty images of each group. (P<0.05, Scale bar=25 μm) $^{*}P$<0.05 compared with sham. $^{**}P$<0.005 compared with sham.

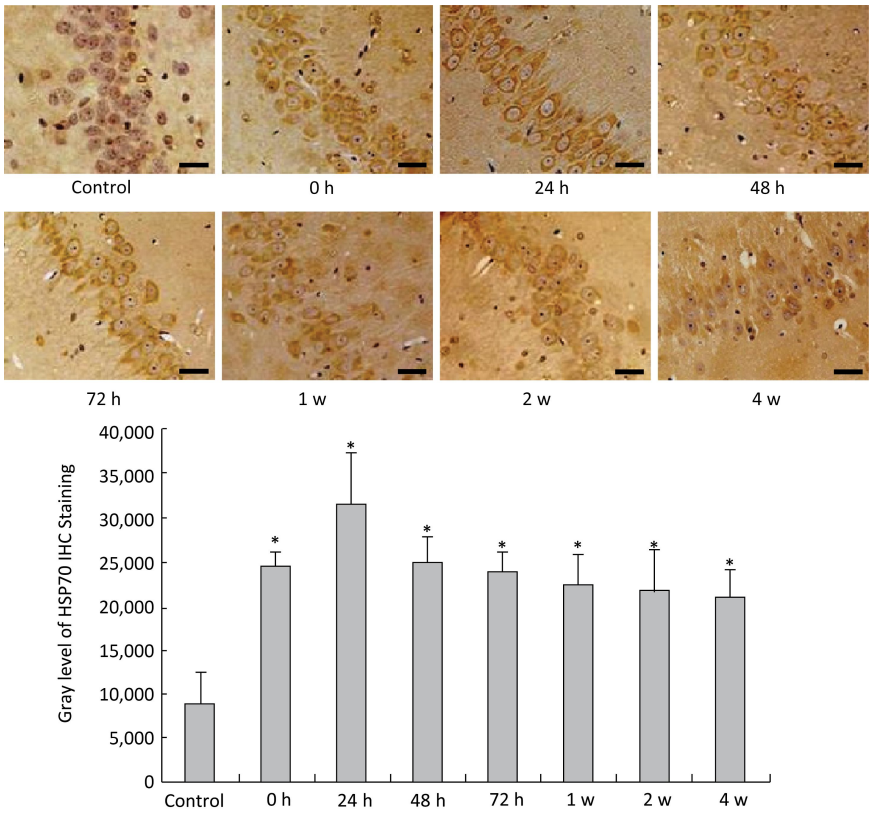

Figure 3. IHC staining of HSP70 in each group. (Scale bar 25 μm). *P<0.05 compared with sham.

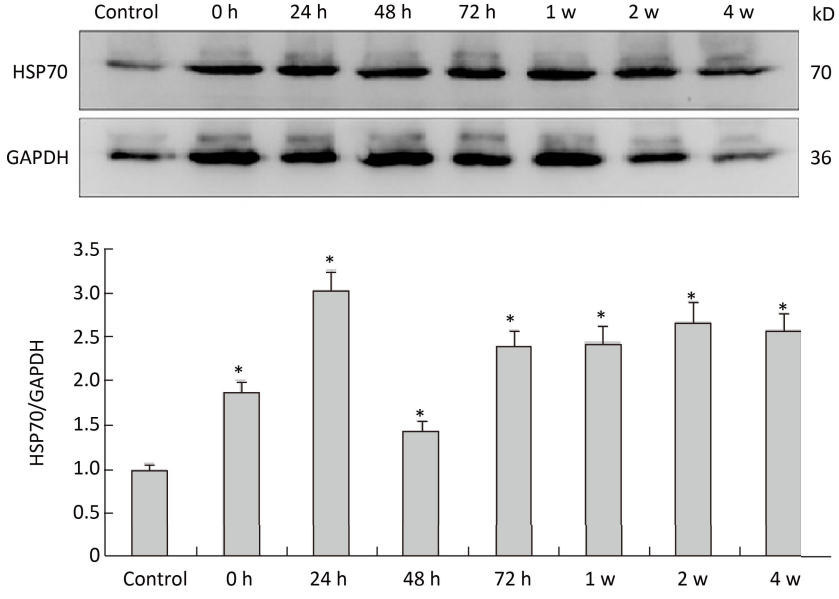

Figure 4. Relative amounts of HSP70 in each group. HSP70 and GAPDH expressed in the hippocampus of rats in the sham group and 0 h, 24 h, 48 h, 72 h, 1 w, 2 w, and 4 w groups after infrasound exposure (8 Hz, 140 dB, 2 h). *P<0.05 compared with sham.

damages to the hippocampus appeared at 0 h and peaked at 24 h, and that recovery began after that. However, damage could be detected even after 4 weeks. These results indicate that exposure to 140 dB (8 Hz) infrasound for just 2 h a day for 3 days can induce morphological damages of the hippocampus and neuronal apoptosis in the hippocampus of rats. Although there was spontaneous recovery from the damage, the damages to the hippocampus of the rats lasted at least 4 weeks.

With respect to hippocampal cell apoptosis, compared with the sham group, neuronal apoptosis in the hippocampus was higher significantly at 24 h and 48 h in rats exposed to infrasound. The analyses also showed that the percentage of apoptotic cells decreased one week after exposure. The results indicate that the infrasound induces injury to hippocampal neurons. However, recovery from the injury appears to occur with the passage of time.

We suggest that the damages induced by infrasound at the molecular and cellular level are caused by resonance from the tissue level to the molecular level. Previous research demonstrated that the resonance of infrasound frequencies close to the human body's vibration, which is between 2 and 16 Hz, will induce tissue damages[18]. The frequency of the human brain's vibration is between 8 and 12 Hz, which is why a frequency of 8 Hz was used in this study[16]. We observed that this frequency resonates with the hippocampus, and thus, causes damage to the hippocampus. The apoptosis pathway of hippocampal cells also was triggered. It is widely acknowledged that cell apoptosis reflects a spontaneous process of cell death that is triggered by various signals. This process is important for the multicellular organisms to develop, survive, and maintain the body in a normal state. Normally, the human body uses this mechanism to complete tissue degradation, and to eliminate impaired, aged, and mutant cells. If this process gets out of control, a disease may appear. Various factors can initiate cell apoptosis, such as DNA damage[19]. Consequently, whether the cell apoptosis induced by infrasound is universal, or tissue specific, needs to be studied further. The hippocampus, with its related structures, is one of the most important parts of the brain. Hence, increased hippocampal cell apoptosis may cause brain dysfunction[20]. This study may help scientists to understand the learning and memory disabilities of human and animals after exposure to infrasound.

Previous results indicated that HSP70 was expressed by the cortex when rats were exposed to in frasound at 90 dB (8 Hz) 2 h per day for two to three weeks. Immunohistochemistry and Western Blot were used in this study to determine the expression of HSP70 after exposure to an experimental infrasound frequency of 8 Hz, with a sound pressure level of 140 dB, 2 h per day for 3 days.

The heat shock proteins (HSP) are a family of conserved proteins that are ubiquitously expressed. The HSP70s are important for the body's steady state, and help to protect cells from stress[21-22]. When a stimulus exceeds a certain threshold, the gene *hsp70* is activated. After the transcription and translation, the HSP70s are expressed increasingly to protect the cell from the damage induced by the stimulus. Therefore, we consider HSP70 to be one of the best markers for showing the ability of the body to be resistant to harmful influences. Normally, brain tissue expresses only a small amount of HSP70. However, when tissue is damaged, the protein secreted by damaged cells induces an increased expression of HSP70, which protects the neuron. In this study, the experimental group experienced a rapid increase in HSP70 just after the exposure, as well as increased HSP70 at the 24 h, 48 h, 72 h, 1 w, and 2 w, compared to the sham group. HSP70 reached a peak at 24 h, which is synchronous with the damage to the hippocampus; the increased expression was sustained for two weeks. The findings confirmed the damage in the experimental group of rats and suggest that the recovery from the damage to the hippocampus may be due to increased HSP70 expression; that is, HSP70 may contribute to the process of recovery. As a marker of neuronal cell impairment and resistance ability, studying HSP70 expression in the damaged hippocampus of rats after exposure to infrasound may show us a path to the potential treatment of diseases caused by infrasound.

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