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Cochlear Synaptopathy Following Noise Exposure in Guinea Pigs: Its Electrophysiological and Histological Assessments

Parvane Mahdi[1], Akram Pourbakht[1,*], Vahid Pirhajati Mahabadi[2], Alireza Karimi Yazdi[3], Mahtab Rabbani Anari[3] and Mohammad Kamali[4]

[1]Rehabilitation Research Center, Department of Audiology, School of Rehabilitation Sciences, Iran University of Medical Sciences, Tehran, 15459-13487, Iran
[2]Department of Neurosciences, School of Medicine, Iran University of Medical Sciences, Tehran, 14496-14535, Iran
[3]Department of Otorhinolaryngology, Tehran University of Medical Sciences, Tehran, 14197-33141, Iran
[4]Department of Rehabilitation Management, Iran University of Medical Sciences, Tehran, 15459-13487, Iran
*Corresponding Author: Akram Pourbakht. Email: pourbakht.a@iums.ac.ir

Abstract: Exposure to high level of noise, may cause the permanent cochlear synaptic degeneration. In present study, a model of noise induced cochlear synaptopathy was established and the electrophysiological and histological metrics for its assessment was designed. 6 guinea pigs were subjected to a synaptopathic noise (octave band of 4 kHz at 104 dB SPL, for 2-h). The amplitude growth curve of Auditory Brainstem Response (ABR) wave-I and wave-III latency shift in presence of noise were calculated. These indexes were considered in pre-exposure, 1 day post exposure (1DPE), 1 week post exposure (1WPE) and 1 month post exposure (1MPE) to noise. Finally, the samples were histologically analyzed. ABR wave-I amplitude was different between pre and 1DPE (p-value ≤ 0.05). However, at 1WPE, it was recovered at low intensities but at 70 dB SPL and above, the differences persisted even till 1MPE. In masked ABR, the latency shift of wave-III was different between pre and 3 post exposure assessments (p-value ≤ 0.05). Electro-microscopic analysis confirmed the synaptic degeneration, as the ribbons were larger than normal, hollow inside, and spherical and irregular in shape, also, the post synaptic density was abnormally thick and missed its flat orientation. These data revealed that noise at level below that can produce permanent hearing loss, can incur synaptic injury. So, noise is considered to be more damaging than previously thought. Accordingly, designing tools for clinical assessment of synaptopathy is beneficial in comprehensive auditory evaluation of those with history of noise exposure and also in hearing protection planning.

Keywords: Cochlear synaptopathy; noise induced hearing loss; hidden hearing loss; excitotoxicity; hearing protection

1 Introduction

Exposure to high level of noise may cause the permanent synaptic degeneration. As a neurotransmitter, glutamate is the main mediator in the auditory afferent system [1–3]. However, when it exceeded the normal

limit due to the over-activation of the auditory system, the "excitotoxicity" will occur [4–6]. A term that refers to the condition in which the exorbitantly released glutamate takes neurotoxic properties and incurs damage to the pre and post synaptic elements.

Opposed to previously published papers about noise-induced auditory damages, the peripheral synaptic connections are the first locus of injury. This synaptopathy can occur even when the hair cells are intact [5,7,8]. The related pathomechanisms may include oxidative stress, calcium influx and metabolic issues such as intracellular ATP reduction, and can occur in presbycusis, ototoxicity and some other auditory disorders in addition to noise exposure [9]. Surprisingly, this degenerative process will not affect auditory thresholds up to the loss of 80% of synapses. Because the auditory neural fibers (ANF) with low spontaneous rate (SR) which are unessential for auditory threshold are preferentially more vulnerable to the noise [10–12]. However, as these fibers have a wide dynamic range, they are critical in temporal processing. So, after their impairment, some supra threshold auditory processing which relies on distinct morphology and function of synapses will be disrupted.

Additionally, considering the central consequences of this impairment, the silence of these auditory fibers, reduce the relied input to the higher auditory stations. Afterward, the neural compensatory mechanism will occur that eventually lead to an increment of central gain. This central hyper activity may have the functional consequences of tinnitus and hyperacusis in some cases [13–16].

Unlike the overt HL, this "hidden hearing loss" (HHL) is not detected based on conventional hearing tests such as audiometry and threshold based-ABR, but it is expected to be revealed in those tests that exert some challenges on auditory system in which low SR fibers should get on the job [14,17–19].

From the clinical view point, the evidence of hidden hearing loss is based on patient's complain as having hearing difficulty despite normal auditory thresholds and have been estimated at 12–15% in prevalence [8,20–22]. The other functional consequences of this disorder are distraction of auditory detection in noisy situation, problem in auditory localization and abnormality in other auditory skills in which precious temporal processing is needed [8,20–22]. This problem is more common in those who had a history of working in noisy environment.

In animal models, the HHL can be effectively studied through near field physiological test and histological examination. As these invasive tools are not applicable in human subjects, the importance of studying on noninvasive, reliable tests that specifically focused on cochlear synaptopathy is self-evident. Based on the results of such tools the more exact lesion site can be studied and the validation of any therapeutic intervention can be documented. In the present study, we chose to establish a model of noise-induced cochlear synaptopathy in guinea pig and then we designed some electrophysiological metrics for its clinical assessment.

Considering the hearing range and physiology of the auditory system in the guinea pig that is somehow homologous of human specious [23], these data may be useful for simulation of pure cochlear synaptopathy in humans. Also, confirming the sensitivity of such tools in diagnosis of synaptic pathology of an animal model may validate their application in human clinical setting.

Auditory brainstem response (ABR) is a neurological test used to assess different aspect of auditory neural function. Generally speaking, the ABR latency corresponds to the speed of neural conduction and its amplitude is a biomarker of neural fibers amount which synchronously takes a part in response. As in cochlear synaptopathy, the detachment of nerve terminals from inner hair cell (IHC) eliminates the fibers from participation in neural response, it is expected to decline ANF-generated ABR wave-I amplitude. So, the amplitude would be an acceptable tool in the detection of cochlear synaptopathy [3,8,11,18]. Furthermore, considering the increased central gain in synaptopathy, the analysis of wave-V (III in guinea pig) amplitude which originates from rostral (central) portion of auditory nervous system may serve additional diagnostic information.

Another index for detection of synaptic loss is the analysis of ABR wave-V (III in guinea pig) latency in the background of noise. The basis of this criterion rest on the assumption that the low SR fibers are dominant in ABR in noise response but with increased latency. Unfortunately, these fibers are the target of injury during synaptopathy [19,24]. So, in synaptopathic ears suffering from the lack of low SR fibers, the increment in response latency was not seen, but the resistance against noise will be reduced and eventually the response morphology will be disrupted at a lower level of background noise.

For the final approve of cochlear synaptopathy, the ultra-structural observation of synaptic zone through histological approach is beneficial. Transmission electron microscopy (TEM) technique, is used in a molecular biology for determining the fine structure of materials. Through this procedure, the morphology of pre and post synaptic elements such as ribbon, neurotransmitter receptor and other components could be observed.

There are discrepancies between studies considering the characters of noise that could incur cochlear synaptopathy. Also there is doubt about the permanency or temporary of the synaptic lesion and about tools that are applicable in diagnosis of this pathology. So, considering the gap in previously published data, the aim of present study was to develop a guinea pig model of cochlear synaptopathy via noise presentation. Additionally, for comprehensive diagnosis of synaptopathy, electrophysiological parameters related to synaptic function in line with histological evaluation were incorporated. Also, all parameters were evaluated in a range of frequencies and along a period of one month follow up for tracking any possible changes during time.

2 Materials and Methods

2.1 Animals

A total of 6 (regardless of the lost animals due to the unknown reasons) male albino guinea pigs (250–350 g) with normal ABR thresholds at 4, 6, 8, 12 and 16 kHz were enrolled in this study. During the experiment, the animals were housed under a controlled condition from a view point of temperature (22 ± 2°C) and lightening (a half day lightness/a half-day darkness cycle) with the accessibility to the free amount of food and water. All procedures were conducted in adherence with the animal care guidelines of the ministry of health and medical education, with approved ethical code of IR.IUMS. REC.1398.429.

2.2 Noise Exposure

According to the majority of experimental work on noise traumatized rodent, it has demonstrated that the TTS of approximately 30–40 dB is most probably comorbid with a certain amount of synaptopathy [5,7,11,17]. Also, the recovery from TTS has been reported maximally till 3 weeks and the residual HL lasting for more than this period considered as permanent one [25].

After titration of noise level we found that an octave band noise of 4 kHz with 104 dB SPL and with 2-h presentation will induce our intended TTS which was fully recovered in one week follow up. Consequently, the aforementioned noise constituted the synaptopathic noise. The spectrum of the noise was shown in Fig. 1.

The noise was generated within a single-walled, sound-deadened chamber. The level of noise was calibrated with SLM (B & K, model 2243, Denmark). The chamber was designed in 60 × 80 × 100 cm of glass and galvanized iron. The noise was presented with a noise generator (Benaphone Electronic Company, Iran). The noise emanated from the speaker supported with a power amplifier. The distance of the speakers from the ears was 10 cm. During the exposure, animals were awake and kept in a wire-mesh cage. In order to be sure about the reaching of intended intensity to the animal's ear, only one animal was placed in each cage.
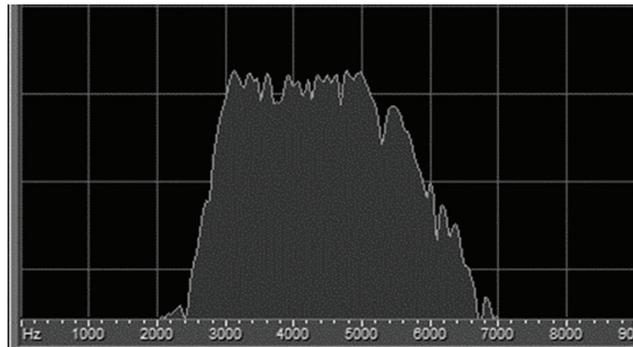
Figure 1: The spectrum of the synaptopathic noise, an octave band noise centered at 4 kHz with dispersion of energy in 3–6 kH

2.3 Auditory Brainstem Response Measurement

Animals were anesthetized with an intraperitoneal injection of ketamine 10% (40 mg/kg, Alfasan, The Netherlands) and xylazine 2% (4 mg/kg, Alfasan, the Netherlands). Body temperature was kept at 37.0 ± 0.5°C using a heating pad. ABR was measured using the Bio-logic device (GN Otometrics, Denmark). Three needle electrodes were placed and fixed subcutaneously on the vertex (noninverted), below the right pinna (inverted) and the contralateral pinna (ground). Interelectrode impedance was kept below 5 kΩ. The acoustic stimuli were alternating tone burst of 4, 6, 8, 12 and 16 kHz presented at a repetition rate of 11.1/sec. Responses to 1024 stimuli were preamplified and bandpass between 100 and 3000 Hz. The analysis time of the screen was 10 ms. The intensity was decreased from 90 dB SPL to the lowest level at which the ABR wave-III, the most robust and repeatable wave in a guinea pig, could be tracked and it was considered as an ABR threshold.

Generally, in ABR analysis the amplitude is very susceptible to be influenced by factors unrelated to neurologic processes such as head size, skull thickness and physiologic noise. For controlling the inter subject variability, the differential measure of wave I is more informative in a clinical setting. However, in rodent electrophysiological assessment because of small head size of animal, anesthesia and muscle relaxant injection, and with sub-dermal needle electrodes, the response can be recorded robustly with high degree of reproducibility, and the exact value of amplitude can be confidently compared between cases. Additionally, at the present study, the duplicated recordings were made at each level of intensity, and just those waves with confirmed reproducibility were taken in to account. The peak-to-trough amplitude of wave I was calculated and for recording amplitude growth curve the stimulus level was varied from 90 to 40 dB SPL in 10 dB step.

For recording masked ABR, the tone burst of 4, 6, 8, 12 and 16 kHz at a constant level of 80 dB SL presented simultaneously with broad band noise extended from 1 to 16 kHz. The level of masking noise was initially set at 0 dB SL and it was considered as the no-noise condition and then it was increased in 10 dB step until the ABR was just masked and no more response could be recorded. At each noise level, the response detectability, the wave-III peak latency and the amount of latency shift was calculated.

All of these tests were repeated in predetermined time points. First, they were done on the pre-exposure to noise in order to define the base line values, then on one day post exposure (1DPE) for determining the effect of temporary threshold shift (TTS) on test results, on one week post exposure (1WPE), after recovering the TTS, for confirming the effect of synaptopathy on tests results and one month post exposure (1MPE) for final approve of synaptopathy and to ensure the permanency of synaptopathy.

2.4 Histological Examination

At the end of our experiment, at 1MPE time point, the animals were sacrificed for providing histological samples. Animals were decapitated under deep anesthesia using a mixture of xylazine and ketamine. For TEM technique, temporal bones of right side were removed immediately, the bony shell of the cochlea was dissected out, the round and oval windows were exposed and the perilymphatic spaces perfused with phosphate buffered saline (PBS), then 2.5% glutaraldehyde was used as a primary fixation for 48 hours. For removal of free glutaraldehyde, the tissue was rinsed 2–3 times with PBS. Then, 1% osmium tetroxide was used as a secondary fixation for 1.5 hours. The tissue was dehydrated in acetone (50%, 70%, 90%, 100%), and infiltrated by resin and finally embedded in pure resin (Epon 812, TAAB, UK). Basilar membrane belonging to 4–8 kHz region (at around 1.5 turn of the cochlea or 10.08–12.65 mm from the apex, based upon the frequency-distance mapping of guinea pig) was sectioned for TEM [22]. Tissue was sectioned parallel to the modiolar axis, first, semi-thin (500 nm) section was prepared and stained with toluidine blue to make the targeted synaptic part identifiable during light microscopy (40–100x). The part of a specimen that belongs to the contact of IHC to auditory nerve terminals was considered as a synaptic zone and used for further consecutive dissections (Fig. 2). Second, thin (50 nm) serial sections were performed for electron microscopy. Thin sections were transferred on the 200-mesh uncoated grids and stained with uranyl acetate and lead citrate before imaging by TEM (LEO 906; Zeiss).
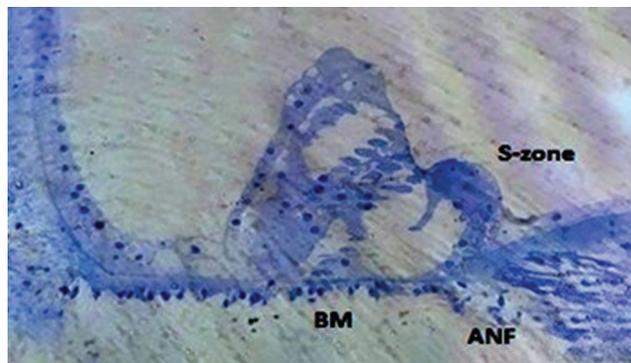

Figure 2: The light microscopic image from semi-thin section of cochlear tissue which was stained with toluidine blue. BM: Basilar Membrane, ANF: Auditory Nerve Fiber, S-zone: synaptic zone

3 Statistical Analysis

SPSS software, version 16 (Chicago, IL, USA) was used for statistical evaluation. Firstly, the normality of the data distributions was examined by calculating the standardized skewness and kurtosis index, in the Shapiro-Wilk test the statistics values varied from -3 to 3 with a p-value of more than 0.05, indicating that the data distributions did not differ significantly from normality. To compare different values between pre and three post-exposure time points, ANOVA with repeated measure test was used. The criterion for statistical significance was defined as p-value ≤ 0.05.

4 Results

4.1 ABR Thresholds

Tab. 1 demonstrates the ABR thresholds of 4, 6, 8, 12 and 16 kHz in all 6 cases. The thresholds (mean ± SD) of baseline (pre-exposure), one day post exposure (1DPE), one week post exposure (1WPE) and one month post exposure (1MPE) were assessed and the comparison between these assessments were done. ABR thresholds in the pre-exposure assessment were essentially equivalent among all animals in each frequencies. The threshold of high frequency tone bursts was lower than the low frequencies, the

Table 1: The Mean ± SD of ABR thresholds in pre-exposure and 3 post noise exposure assessment and their p-values

Frequency (Hz)	Pre-Exp Mean ± SD (dB)	1DPE Mean ± SD (dB) P-Value	1WPE Mean ± SD (dB) P-Value	1MPE Mean ± SD (dB) P-Value
4000	22.5 ± 2.70	57.83 ± 3.76 0.020*	25.0 ± 3.16 0.076	24.16 ± 2.04 0.176
6000	22.50 ± 2.73	65.66 ± 4.08 0.000*	25.0 ± 4.47 0.063	24.16 ± 2.04 0.175
8000	21.66 ± 2.58	53.33 ± 6.05 0.000*	32.50 ± 1.08 0.078	20.83 ± 2.04 0.363
12000	17.5 ± 2.73	24.16 ± 3.76 0.061	19.16 ± 2.04 0.175	18.33 ± 4.08 0.351
16000	16.66 ± 2.58	20.83 ± 2.04 0.083	19.16 ± 2.04 0.203	15.83 ± 2.04 0.363

*Statistically significant

underlying reason was that as the high frequencies are decoded in base of cochlea and they are close to recording electrodes, so they result to sharper responses.

ABR thresholds of 4, 6 and 8 kHz were statistically different between pre and 1DPE (p-value ≤ 0.05), with the greatest amount of threshold shift, about 43 dB, at 6 kHz. The thresholds of 12 and 16 kHz, was not statistically different from base line value (p-value > 0.05), so these two frequencies were excluded from our further analysis.

There were no significant differences between the pre-exposure values with 1WPE and 1MPE at neither frequencies (p-value > 0.05), suggesting that the noise exposure did not produce any PTS at all.

4.2 ABR Wave-I Amplitude Growth Function

Fig. 3, presents the amplitude growth curve of all tone bursts. As it was shown, the largest amount of amplitude reduction due to the noise exposure was noted in 1DPE and it was followed by partial recovery in 1WPE assessment, however there was almost no more significant recovery in 1MPE.

The biggest amount of amplitude reduction was noted at 6 kHz, in which the most amount of TTS was recorded too. The comparison of amplitude at 90 dB SPL in pre-exposure (3.68 ± 0.34 µV) with 1DPE (1.77 ± 0.32 µV) revealed a reduction to 48.09% that was recovered to 63% in 1WPE (1.32 ± 0.54 µV) and with little more recovery in 1MPE (2.61 ± 0.24 µV) to 70%.

A repeated measures ANOVA was performed against pre and all three post noise exposure assessment and Tab. 2, demonstrates the relevant results. As it was shown, there were statistically significant differences between pre-exposure and 1DPE amplitude at all intensity in 4, 6 and 8 kHz (p-value ≤ 0.05), however in 1WPE assessment due to the partial recovery, the amplitude was increased and there was no more statistically different values at low intensities but at 70 dB SPL and above, the differences persisted and it wasn't recovered even at 1MPE, suggesting the permanency of this damage.

4.3 ABR Wave-III Latency Shift in Noise

At noise level below 30 dB SL there was no obvious effect of masking noise on latency of the response. However, above 30 dB SL the latency was increased. As at 80 dB SL noise level, signal to noise (S/N) ratio of 0, the response was not detected anymore, the noise of 70 dB SL set the highest level of our applied noise. Fig. 4, depicted the ABR in the noise of one of our cases in the pre-exposure assessment.

In pre-exposure assessment the response was detected even at the S/N ratio of 10, meaning that the background noise level of till 70 dB SL was tolerable for ABR recording. In 1DPE assessment due to the hearing loss the presentation of tone burst and noise at high level was impossible, so the masked ABR
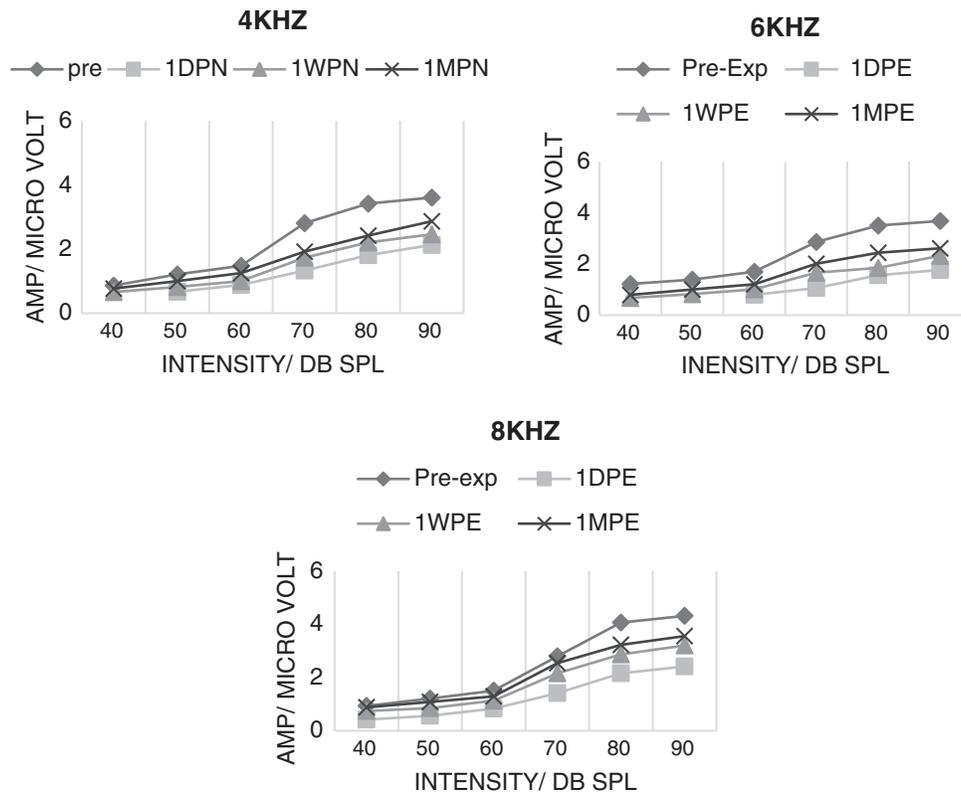
Figure 3: ABR wave-I amplitude growth cure in 4–8 kHz tone bursts. The greater amount of amplitude at high intensities and less steeper curve in all post-exposure time points

could not be recorded on this time point. In 1WPE and 1MPE assessment the response was tracked down just till 50–60 dB SL noise level. Fig. 5 illustrates the amount of wave-III latency shift as a function of noise level.

Considering the amount of latency shift of wave III, there were statistically differences between pre and all post-noise exposure assessments (p-value ≤ 0.05), with the most significant at higher noise intensity. Post hoc analysis revealed no differences between 1DPN, 1WPN and 1MPN, meaning that the effect of synaptopathy on this parameter was permanent during a period of follow up. Tab. 3, demonstrates the details of this statistical analysis.

4.4 Histological Findings

For final approval of synaptopathy, the cochlear tissue of our samples was histologically examined by TEM. The ultra-structural features of synaptic elements were traced and compared with intact ones. As it was shown in Fig. 6, the size of the synaptic ribbon in the experimental group was found larger than that of their intact counterpart. This increment of ribbon size was documented by larger cross area of ribbon existing sections. Also, these swollen ribbons were hollow too, with less mass density inside, meaning that they lost their intra cellular components and it was evident by brightness of image on inner part of ribbons. The other differences compared to the intact samples was that the ribbons lost their round shape and became somehow spherical with a little irregularity in shape. Additionally, some diversity was also noted on post synaptic part. The post synaptic density (PSD) was thicker in noise traumatized group and it missed its normally flat orientation and became deviated.

Table 2: The statistical comparison of ABR wave-I amplitude between pre-exposure and post noise exposure assessment in different frequencies and intensities

Frequency (Hz)	Intensity (dB SPL)	1DPE p-Value	1WPE p-Value	1MPE p-Value
4000	40	NR	0.176	0.885
	50	0.013*	0.243	0.335
	60	0.037*	0.075	0.629
	70	0.006*	0.002*	0.047*
	80	0.001*	0.002*	0.003*
	90	0.002*	0.006*	0.016*
6000	40	NR	0.018*	0.053
	50	NR	0.12	0.262
	60	0.043*	0.032*	0.054
	70	0.000*	0.000*	0.003*
	80	0.000*	0.000*	0.001*
	90	0.000*	0.000*	0.000*
8000	40	NR	0.000*	0.075
	50	0.001*	0.045*	0.063
	60	0.002*	0.214	0.43
	70	0.000*	0.000*	0.006*
	80	0.000*	0.002*	0.036*
	90	0.000*	0.002*	0.008*

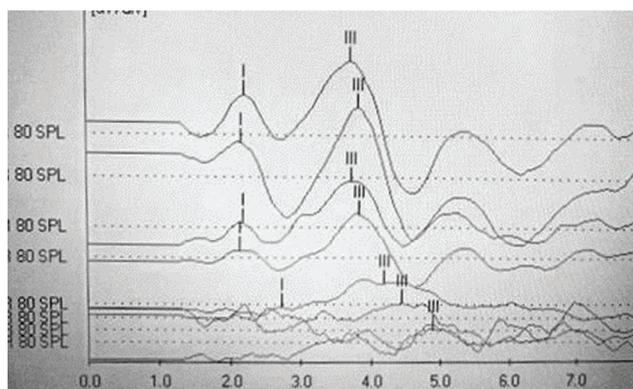*NR: No Response*
* Statistically significant

Figure 4: ABR in noise of 6 kHz tone burst at 80 dB SPL and different level of background noise

5 Discussion

In the current study, the notion of synaptic injury precedence to hair cell loss in noise exposure condition was supported through designing a successful synaptopathic model by noise, evaluated by ABR and
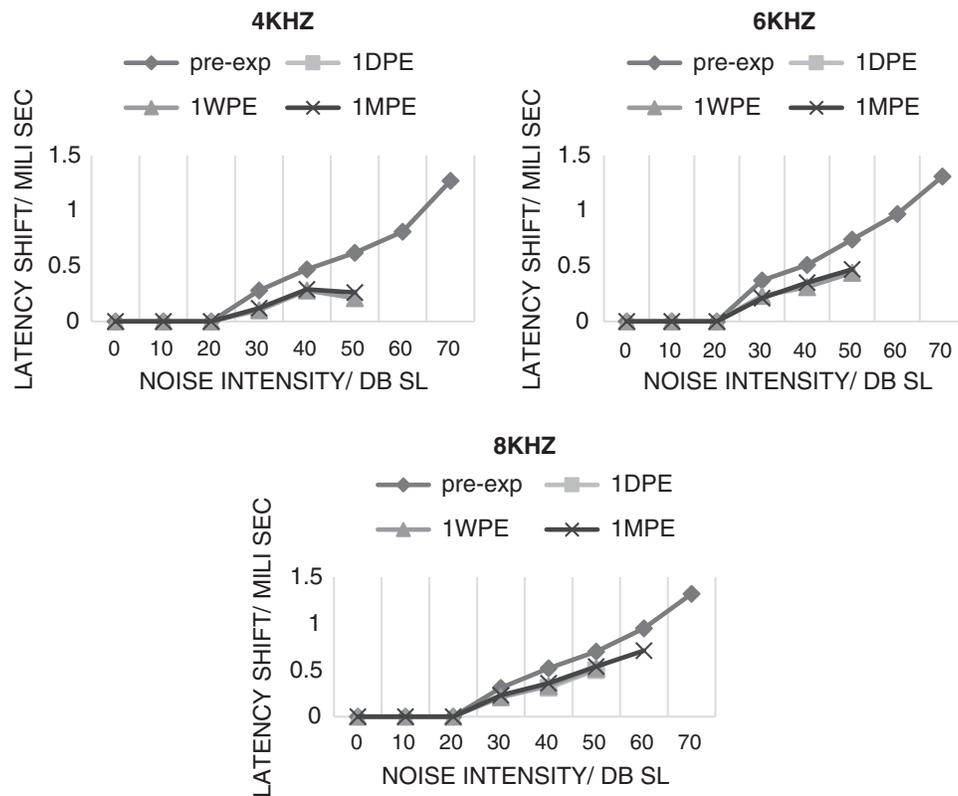
Figure 5: The wave III latency shift as a function of noise level. At post noise exposure, the latency increment was less than pre-exposure time point with reduced response record ability at high noise level

confirmed by histology. Cochlear synaptopathy following noise exposure, was first described by Kujawa and Liberman (2009) [26]. It was first performed on rodent samples and was then examined on other species such as cats, chinchillas and macaques [27–29,32]. Furthermore, it was also have a probability in human with history of life time exposure to noise in spite of normal auditory thresholds [8]. Additionally, this de-afferentation of synaptic connection was also noted in a range of sensoryneural HL such as presbyacousis, ototoxicity and aging [5].

In present study, the synaptopathic model was done using a definite noise and the resultant synaptic loss was persisted up to one month follow up. It should be noted that, as we incorporated an octave band noise of 4 kHz, the resultant HL in 1DPE was mostly in 6 kHz with fewer HL in adjacent frequencies of 4 and 8 kHz. Considering the spectrum of a noise which had an energy dispersion in 3–6 kHz range, with a peak in 4 kHz, and as the basilar membrane vibrations show maximum displacement at half an octave over the offending frequency, this pattern of HL was reasonable.

In a first report of synaptopathy which was done on CBA/CaJ mice, at intensity level similar to what was used in a present study, a moderate TTS, that is mean 40 dB HL, was occurred and up to half of IHC/SGN synapses were reported to be lost and it persisted despite a full recovery of auditory thresholds with no sign of synaptic regeneration in 8 weeks follow up [26].

In an immunohistochemistry (IHC) examination, Shi et al. [17], reported that the repair of synapses will occur if the recovery time extended to 4 weeks. However it seem that the recovery was due to the up and down regulation of pre and post synaptic proteins found in the immunostains, rather than degeneration and regeneration of the synapses themselves.

Table 3: The statistical comparison of the amount of ABR wave-III latency shift in noise between pre-exposure and 3 post noise exposure assessment

		1DPE	1WPE	1MPE
Frequency (Hz)	Noise level (dB SL)	*p*-Value	*p*-Value	*p*-Value
4000	30	NR	0.091	0.088
	40	NR	0.007*	0.007*
	50	NR	0.000*	0.000*
	60	NR	NR	NR
	70	NR	NR	NR
	80	NR	NR	NR
6000	30	NR	0.040*	0.040*
	40	NR	0.000*	0.000*
	50	NR	0.000*	0.000*
	60	NR	NR	NR
	70	NR	NR	NR
	80	NR	NR	NR
8000	30	NR	0.118	0.122
	40	NR	0	0.002
	50	NR	0	0
	60	NR	NR	0.003
	70	NR	NR	NR
	80	NR	NR	NR

NR: No Response
* Statistically significant

For examining synaptopathy in non-human primate, Valero et al. [28] studied on the effects of acoustic overexposure on the rhesus macaques. They reported that exposure to narrowband noise of 108 dB SPL for 4 h, leads to the slight TTS of about 20 dB with moderate synaptic loss of 12–27% and for more synaptic loss of about 50% through histology, the higher noise level that will produce PTS is required. So, from their final conclusion it can be inferred that primate are less vulnerable to noise than rodent but with the same pattern of synaptopathy and later injuries.

There are some evidences supporting cochlear synaptopathy in human subjects. In a recent study done by Dewey et al. [30], they incorporated functional magnetic resonance imaging (fMRI) from central auditory pathway of group of subject with normal audiogram but with positive history of lifetime noise exposure. They found the enhanced fMRI response compared to low noise exposure group. Their results suggest that noise exposure may be associated with central hyperactivity. This phenomenon is most probably due to the synaptic loss and reduction of peripheral input to higher auditory stations.

In an attempt to look for a sign of cochlear synaptopathy in human, Liberman et al. [31], incorporated 2 groups of participant as low and high risk according to their self-report of noise exposure. In addition to reduction of auditory nerve response amplitude in high risk group, they showed significantly poorer performance on word recognition in noise or with time compression and reverberation, and also
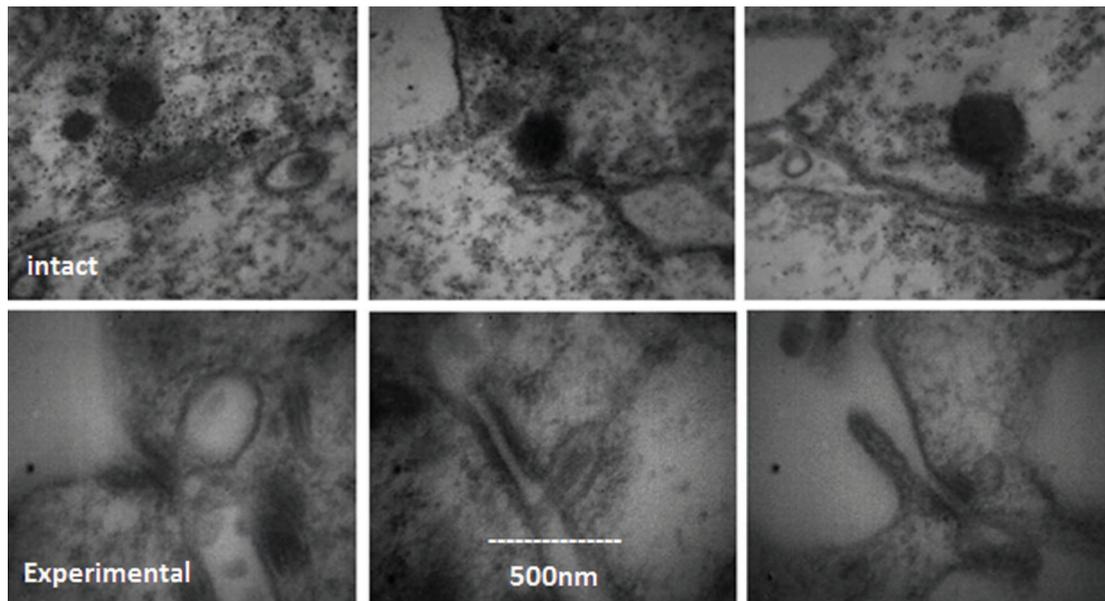
Figure 6: Representative transmission electron micrographs from intact and experimental groups. The electron micrographs showed size, shape of synaptic ribbon and post synaptic density (PSD)

overreaction to high level sound that consisted with hyperacusis. Their final conclusion was that in human with cochlear synaptopathy, in spite of abnormal electrophysiological parameters related to synaptic function, some deficits can be recognized in their auditory performance in difficult listening situations. Although, these results are in favor of confirming cochlear synaptopathy in human, for direct conclusions, the post mortem examination of temporal bone in highly needed.

The reduction of ABR wave I amplitude has been confirmed in synaptopathic model in some studies and often times it did not return to its base line value in spite of threshold recovery, meaning the permanency of synaptopathy [11,19,29].

Concomitant to previously reported article, in present study we observed the reduction in ABR wave-I amplitude following noise induced synaptopathy. The underling neurophysiological basis for this finding is that, following synaptic damage and due to the injury of low SR neural fibers which are responsible for listening at supra threshold levels, the amplitude of ABR will be reduced at high intensity stimuli. In this regard, the pre noise exposure assessment revealed steeper wave-I growth curve (e.g., at 6 kHz, in pre exposure assessment, the slop of amplitude growth curve of wave I was about 0.56 which was higher than the same value at 1DPE, 1WPE and 1MPE which was 0.34, 0.39 and 0.41, respectively) that means after noise exposure the growth of amplitude at high stimulus level is reduced.

Interestingly, this pattern of amplitude growth function persisted even after accounting for TTS, suggesting that although the partial restoration of malfunctioned synapses are enough for auditory thresholds but these survived synapses are not adequate for higher auditory processing in which more precious morphology of synapses are necessarily needed.

In a study done by Lobarinas et al., a reduction of wave I amplitude was documented in rat model of synaptopathy, but they did not find any correlation between this finding and the score of speech in noise detection [11]. However, as hearing skills in noise are somehow multi factorial function that requires not only intact synaptic operation but also other cognitive process, so their results should be interpreted with caution.

The reduction of ABR wave I amplitude was also confirmed in human with history of life time noise exposure in spite of normal auditory thresholds such as military veterans [8].

Considering the amplitude of wave-III, there was no reduction in this parameter. Indeed, following reduction of peripheral input due to the synaptic loss, the compensatory mechanism will take part and central gain will go up which eventually leads to normal or even enhanced higher level auditory response. Surprisingly, the same pattern of finding was seen in some tinnitus patients with normal audiogram. So, one of the probable perceptual consequences of synaptopathy may be tinnitus due to the hyperactivity of auditory central network [14,33].

In line with our finding, Mohele et al. [13], exposed 3 age group of rats, including young, middle aged and old animals to an acoustic overstimulation in which synaptopathy was occured (8–16 kHz, 100 dB SPL, 2 h). They noted that, following nerve silence caused by synaptopathy, the late ABR waves appear with shorter latency and increased amplitude, however, old animals did not reach to this neural compensatory because of reduced central plasticity.

The first report of noise effects on ABR latency increment goes back to 1983, in which, Burkard et al. [24], reported an increase in I-V latency interval in masked ABR. For the explanation of neurophysiological basis of this finding it should be noted that as in our study and that of Burkard, during masked ABR, the increase of stimulus rate did not lead to more increase in latency, so the mechanism of both rate increment and noise presentation are the same and are of neural mechanism. From our results it can be deduced that as in synaptopathic process the delayed low SR fibers are firstly targeted, the prolongation of wave III latency in masked ABR was reduced, instead, the detectability of the response was lost at lower level of noise. It means that, the maximum tolerable noise was 70 dB SPL in pre exposure time point and it drop to 50–60 dB SPL in post noise exposure assessments. For justification of this phenomenon it can be said that high SR fibers, are the only intact neural fibers in synaptopathic cases. Unfortunately, these fibers have a restricted dynamic range and quickly reach to their maximum discharge rate in noise. Additionally, the hearing in noise is mostly depends on detection of fine structure of a tone which is a kind of temporal processing skills, again the low SR fibers cannot do this process so well due to their limited DR [33], so these fibers are not suitable for hearing in noise function.

In accordance with our finding, Mehraei et al., evaluated the latency shift of wave V in presence of noise in human cohort, they found significant relation between the amount of latency shift and perceptual temporal processing: human subjects with the worst sensitivity to envelope interaural time difference (ITD) showed the smallest slope relating wave-V latency to noise level. Additionally, they reported that the slop of wave IV latency in mice with noise induced synaptopathy was smaller than normal cases and this index was in compliance with reduction of wave I amplitude in diagnosis of cochlear synaptopathy [19].

So, according to their finding and that of present study, the application of masked-ABR as a useful metric in detection of synaptopathy is justifiable.

For structural approval of synaptopathic model, the different histological methods have been incorporated till now. Paquette et al. [29], applied a synaptopathic noise to mice and utilized immunofluorescence and confocal microscopy for documentation of synaptic loss. For identifying orphan or unpaired synapses, they considered one-to-one pairing of Ctbp2, the presynaptic ribbon marker, and Gria2, the post-synaptic receptor marker. They noted the significant incompatibility between these values and a related reduction of 37.5% in synaptic number.

In final step of our study, the synaptopathic model was confirmed by histological observation. The ultra-structural properties of our experimental samples were different from intact ones. In compliance with our study, Song et al. [35], incorporated TEM for tracking changes in ribbon synapses morphology following exposure to synaptopathic noise. They reported larger size of ribbon at modiolar side of cochlea that

belongs to low SR fibers, additionally low density space inside the ribbon, swollen post synaptic terminals, increase of synaptic vesicles and deviation of PSD angle were also reported.

In summary, we have presented evidence that implies the presence of cochlear synaptopathy in cases exposed to damaging level of noise. According to most federal guideline in the field of noise protection, the exposures that did not produce any threshold shift are considered safe because the hair cells are intact. However, considering recent animal study, it is now proposed that noise level less than the cause of threshold shift, may lead to primary synaptic pathology with subsequent auditory processing disorders. So, noise is more damaging than previously thought and designing diagnostic tools for identification of related injury and planning for its prevention may be beneficial, also, it would be noteworthy that in planning hearing protection program, the industrial environment with noise level that may result to cochlear synaptopathy should take in to account, too. Additionally, for complete assessment of noise exposure effects, applying the tests that can detect the cochlear synaptopathy are so practical.

For further study and to overcome the limitation of present study it is recommended to establish cochlear synaptopathy model on larger sample size and also extending the follow up time. Also it is useful to incorporate other audiological tests that are sensitive to synaptopathy such as frequency following response (FFR) to comprehensive evaluation of synaptopathy.

Conflicts of Interest: The authors have nothing to declare.

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