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The human neuroprotective placental protein composition suppressing tinnitus and restoring auditory brainstem response in a rodent model of sodium salicylate-induced ototoxicity

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ABSTRACT

The effect of neuroprotective placental protein composition (NPPC) on the suppression of tinnitus and the restoration of the auditory brainstem response (ABR) characteristics was explored in tinnitus-induced rats. The animals were placed into two groups: (1) the study group, rats received sodium salicylate (SS) at the dose of 200 mg/kg twice a day for two weeks, and then 0.4 mg of the NPPC per day, between the 14th and 28th days, (2) the placebo group, rats received saline for two weeks, and then the NPPC alone between the 14th and 28th days. The gap pre-pulse inhibition of the acoustic startle (GPIAS), the pre-pulse inhibition (PPI), and the ABR assessments were performed on animals in both groups three times (baseline, day 14, and 28). The GPIAS value declined after 14 consecutive days of the SS injection, while NPPC treatment augmented the GPIAS score in the study group on the 28th day. The PPI outcomes revealed no significant changes, indicating hearing preservation after the SS and NPPC administrations. Moreover, some changes in ABR characteristics were observed following SS injection, including (1) higher ABR thresholds, (2) lowered waves I and II amplitudes at the frequencies of 6, 12, and 24 kHz and wave III at the 12 kHz, (3) elevated amplitude ratios, and (4) prolongation in brainstem

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transmission time (BTT). All the mentioned variables returned to their normal values after applying the NPPC. The NPPC use could exert positive therapeutic effects on the tinnitus-induced rats and improve their ABR parameters.

Abbreviation

Full Name	
NPPC	Neuroprotective Placental Protein Composition
ABR	Auditory Brainstem Response
SS	Sodium Salicylate
GPIAS	Gap Pre-Pulse Inhibition Of The Acoustic Startle
PPI	Pre-Pulse Inhibition
BTT	Brainstem Transmission Time
AEPs	Auditory Evoked Potentials
PVCN	Posterior Ventral Cochlear Nucleus
AVCN	Anterior Ventral Cochlear Nucleus
LL	Lateral Lemniscus
IC	Inferior Colliculus
AN	Acoustic Nerve
HPE	Human Placental Extract
XP	X-Proteins
CAP	Compound Action Potential
NIH	National Institutes Of Health
IP	Intraperitoneal
SR	Startle Reflex
SPL	Sound Pressure Level
BBN	Broadband Noise
kΩ	Kiloohm
SPSS	Statistical Package for Social Sciences
SD	Standard Deviation
BTT	Brainstem Transmission Time
NMDA	N-Methyl-D-Aspartate
GABA	Gamma-Aminobutyric Acid

1. Introduction

Tinnitus represents one of the common disorders of the auditory system, which can be defined as auditory perceptions without external audit sources, but expressed as phantom ones [1,2]. Tinnitus significantly burdens the healthcare system by affecting 5.1% up to 42.7% of the adult population. The exact pathophysiology of the subjective idiopathic tinnitus generation has remained unclear, and no particular effective treatment has been so far specified [3–6]. Previous research has further demonstrated the importance of the central auditory pathways, spanning the cortical layers and the subcortical structures, such as the hypothalamus, the hippocampus, and the amygdala in the brain [7–10].

One of the commonly approved behavioral methods to identify tinnitus as well as estimate hearing impairment in experimental studies would be the use of the gap-prepulse inhibition model of acoustic startle reflex (GPIAS) and pre-pulse inhibition (PPI) of the acoustic startle reflex (ASR) methods [11]. The GPIAS paradigm can be performed for detecting tinnitus caused by drug-induced ototoxicities such as sodium salicylate or loud noise exposure. GPIAS consists of two phases, with and without a silent gap presented before the start pulse under the background noise environment. In normal conditions, a silent gap inhibits the startle reflex. In animals with tinnitus, disinhibition of silent gaps indicates the presence of tinnitus due to the filling of the gaps with the tinnitus signal. In parallel to GPIAS, Pre-pulse Inhibition (PPI) is performed to evaluate if the rats can detect and hear the background noise in the GPIAS test. The PPI consists of two random phases, single or by pre-pulses before the startle stimulus. When the startle stimulus is presented in a silent environment, the Startle's response increases. The startle response is reduced when a pre-pulse is given before the startle stimulus. Tinnitus is considered when the GPIAS result is unsuccessful, but the PPI test is positive [12]. The auditory brainstem response (ABR) test, also known as the auditory evoked potentials (AEPs), has been accordingly documented for measuring hearing threshold as an evoked potential recording method, generally practiced to reflect on the auditory functions under either pathological or healthy conditions. In this respect, the high temporal resolution (<1 ms) of the AEPs provides a suitable method to take track of the electrical brain activities (viz., the waves), time-locked to the auditory events. The majority of previous studies on rodent ABRs have

reported that the ABR wave I originates from the proximal portion of the cranial nerve VIII, wave II is generated by the posterior ventral cochlear nucleus (PVCN), wave III arises from the anterior ventral cochlear nucleus (AVCN) and the trapezoid body, wave IV comes from the superior olivary complex (SOC), and wave V is attributed to the lateral lemniscus (LL) and the inferior colliculus (IC) [13,14].

On the other hand, the ABR amplitudes for waves II, III, and V may produce data concerning the ABR generators or modulators across the auditory brainstem areas [15]. In contrast, the ABR latencies for waves I–V provide evidence of auditory brainstem function [16]. Research animal studies have further presented the ABR test as an objective technique to show the auditory system alterations, which can represent the existence of tinnitus, causing lower sensory transmission and offsetting the changes in the central structures [17,18].

Nonetheless, tinnitus induction is typically associated with pathological activities in one or more cranial nerves, especially the auditory ones. In this line [19], found behavioral evidence suggesting that the damage to rats' acoustic nerve (AN) fibers could be related to tinnitus perceptions. Other studies have correspondingly shown that tinnitus is correlated with cochlear synaptopathy, the non-appearance of any evident damage to the cochlear function in subjects without peripheral hearing loss, and the AN feedback depletion due to different high-threshold AN fibers. Furthermore, neural plasticity in response to masking hearing loss can lead to patterns of pathological activity in the auditory brainstem that potentially cause tinnitus [20,21].

Salicylate is one of the metabolites of Aspirin (Acetylsalicylic acid) and has been shown to cause auditory abnormalities in humans [22] and animals [23]. It is stated that a high dose of Salicylate creates tinnitus and hearing loss [24]. According to reported studies, chronic tinnitus induced by Salicylate could be associated with extensive changes in electrophysiological, histological, and molecular levels leading to other impairments such as degeneration, necrosis, apoptosis, etc., in neurons and brain tissue. Given the above information, if any agent can help decrease these impairments, it would also improve tinnitus [25,26].

Given the existing literature on the pathophysiology of tinnitus and the ineffectiveness of current treatments for this condition, it is of utmost importance to provide evidence-based, rational therapeutic interventions.

The placenta is an organ that temporarily connects the developing fetus to the mother and is rich in various bioactive substances, including proteins, minerals, amino acids, nucleic acids, growth factors, cytokines, enzymes, vitamins, and trace elements [27,28]. The animal and human placental extract (HPE) preparations have been accordingly exploited in different parts of the world as traditional, complementary, and recently, regenerative medicines for prophylactic and therapeutic purposes, such as wound healing, aging protection, organ protection, radioprotection, neuroprotection, neuro-rehabilitation, etc. [29,30]. Although studies on the specific functions of HPE components have been scarce, findings suggest that various HPE proteins and peptides are involved in the processes of the oxidative stress response, resolution of inflammation, and tissue regeneration, which can be further used in modern medicine [31–33].

Some studies have thus reported the beneficial neuroprotective effects of the HPE and the HPE protein components in the animal models of chronic stress [34], hypoxia/ischemia [27], and Alzheimer's disease [35], leading to cognitive and memory function improvements.

Similarly, the protein fraction of the porcine placental extract was introduced by Alexander Anikin to have prominent neuroprotective properties [36]. They further reported the evident positive outcomes of the preparation, initially named X-proteins (XP), on several brain stroke and trauma patients with lasting severe neurological defects, for whom the daily intranasal XP administration during a 12-to-14-day treatment period had reduced the patients' motor, mental, and speech deficits, and improved their emotional states. They concluded that XP might induce the regenerative/reparative processes in the reversibly damaged nervous tissues [36].

According to the possible neuroprotective effects of the HPE mentioned above, and due to the various mechanisms involved in tinnitus, there is still no proven treatment for it [37]. Therefore, this multi-component composite may be effective in treating tinnitus. To the best of our knowledge, the effect of HPE on tinnitus treatment has not been reported before.

Based on the scarcity of the recommended effective medications for the management of tinnitus and the biological activity of the HPE-derived XP, termed the neuroprotective placental protein composition (NPPC), was described earlier, this study aimed to characterize the Sodium Salicylate (SS)-induced tinnitus alterations of the auditory behaviors and the ABR parameters along with the effects of the NPPC administration on tinnitus suppression in a rodent model of SS-induced ototoxicity.

2. Materials and methods

2.1. NPPC preparation

The human placenta for the NPPC preparation was procured from SinaCell Company, officially certified by the Food and Drug Agency of Iran, for ethically distributing the donated human placenta and placental derivatives for medical and research use. The non-hydrolyzed protein extraction and the NPPC preparation from the human placenta were also performed according to the methodology described by Ref. [36] with minor modifications, including the replacement of the DE-52 cellulose resin (CA. b45059) with the DEAE-Sepharose Fast Flow resin (CA.17070901, Cytiva, the United States) without further size-based fractionation of the obtained protein composition. The final total protein concentration (measured by the QuBit 3.0 fluorometer, CA. Q33216, Life Technologies, the United States) and pH were thus set at ~1 mg/ml and ~7.0, respectively.

2.2. Animal handling and experimental design

In total, 16 male Wistar rats weighing 200–350 g were recruited in this study. The animals were thus housed based on two to three

rats per cage with 12-h light/dark cycles and standard food and water ad libitum freely. Room temperature and humidity were controlled at the favorable ranges of 21–22 °C and 50–55%, respectively. All the procedures were also performed under the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978) and then approved by the Vice-Chancellor's Ethical Committee for Medical Research, Iran University of Medical Sciences, Tehran, Iran (Ethical approval no. IR.IUMS.REC.1398.233).

The given animals were randomly placed into two groups: the chronic study group and the control group. Fig. 1 shows the course and the timeline of the studies. The chronic study group first underwent the behavioral and ABR tests (Sections 2.4 and 2.5) for the baseline evaluations before receiving SS. The tests were then repeated on day 14. From the 14th to 28th days, all rats received the NPPC treatment daily, and SS was given simultaneously for the stability of tinnitus in the animals. On the 28th day, the behavioral and ABR tests were further repeated to evaluate the therapeutic effects of the NPPC (n = 8 rats). The control group also received normal saline for the first two weeks and the NPPC for the second two weeks (n = 8 rats).

2.3. Animal treatment regimens

SS (CAS 54-21-7, Merck, Darmstadt, Germany) was dissolved in saline with a 200 mg/ml concentration. On the first 14 days, the chronic study group received SS (200 mg/kg), twice a day, at 8:00 a.m. and 4:00 p.m., via an intraperitoneal (IP) injection. From the 14th to 28th days, they also received 0.4 mg/kg of the NPPC once a day in addition to SS. The control group then received normal saline (IP, 200 mg/kg), twice a day, for 14 consecutive days, and then received the IP injections of NPPC (instead of the normal saline) once a day from the 14th to 28th days. Both the chronic study and control groups also underwent behavioral tests and ABR.

2.4. Behavioral tests for tinnitus

The GPIAS and the PPI behavioral tests evaluate tinnitus in animals [38]. The present study used an acoustic Startle Reflex system (SR-LAB system - San Diego Instruments, San Diego, Canada) to assess tinnitus. The PPI test and the GPIAS were further considered to determine hearing impairment. The GPIAS and PPI tests were accordingly completed at three-time stages, viz.

(1) one day before the SS injection (at the onset of the study), (2) 14 consecutive days after the SS injection (on the 14th day), and (3) 14 consecutive days after the NPPC and SS injections (on the 28th day). The rats were placed in a sound-attenuating chamber on a piezoelectric platform and kept inside a plastic holder. The downward force created by the movement of the rats on the piezoelectric plate and the analogue-to-digital conversion was also started using the SR-LAB software (San Diego Instruments, San Diego, Canada) to calculate the GPIAS and PPI parameters (Fig. 2).

The peak-to-peak startle amplitude response values were then calculated offline. Two speakers located on the chamber's ceiling ultimately generated the acoustic startle stimuli and the white noise background.

The GPIAS test was accordingly administered using the 60 dB sound pressure level (SPL) background broadband noise (BBN), consisting of 16 trials with a gap and 16 trials without a gap. A 20-ms main pulse burst of the white noise was then performed at the intensity level of 115 dB to get a startle response. The trials with a gap started at 100 ms before the onset of the startle bursts and lasted for 50 ms with a 1-ms rise and fall time. The interval between each startling noise was considered 20 s, and each test took approximately 25 min.

The PPI of the acoustic startle reflex method comprised 15 pulse trials, eight no-stimulus trials, and ten pre-pulse trials. The startle stimuli were presented randomly [namely, single or by a 60-dB SPL BBN burst pre-pulses with 50-ms duration, 1-ms rise/fall time]. The frequency and intensity levels exploited in the PPI test were similar to those in the GPIAS test parameters. Besides, there was a time interval of 100 ms between the noise burst pre-pulse onset and that of the startle stimulus [28].

In the present study, the GPIAS values were calculated based on the following formula, which was by Turner et al., 2006 [28]:

[(AvgTnogap–AvgTgap)/AvgTnogap × 100%]

The AvgTgap represents the average amplitude during the gap trials, and the AvgTnogap indicates the average amplitude of the no-gap test trials. Moreover, the PPI values were computed based on the following formula:

[(AvgTstartle–AvgTpre-pulse)/AvgTstartle × 100%]

The AvgTpre-pulse and the AvgTstartle refer to the average amplitude during the pre-pulse trials and the average amplitude of the startle trials, respectively [28].

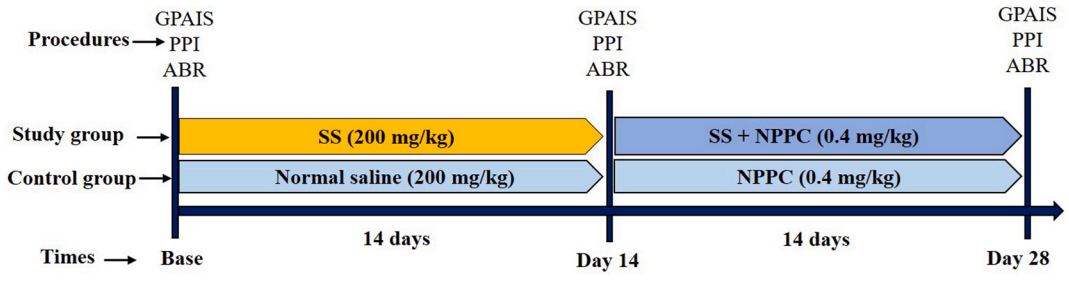

Fig. 1. Experimental design and timelines for the course of the experiment on two animal groups SS stands for sodium salicylate.

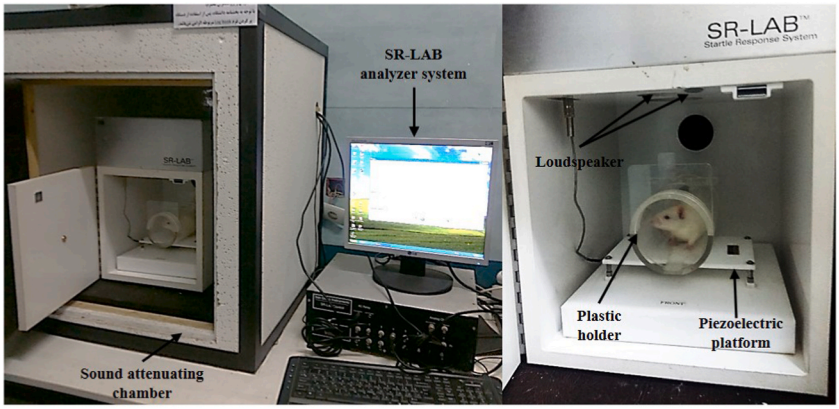

Fig. 2. Acoustic Startle Response (ASR) setup: A behavioral test to assess GPIAS and PPI in rats. The rats were placed in a sound-attenuating chamber on a piezoelectric platform, which was kept inside a plastic holder. The sounds were delivered through two loudspeakers placed on the ceiling of the room, one for background noise and the other for the presentation of the startle stimuli. The arrows show different parts of the setup.

2.5. ABR measurements

The ABR was recorded in a soundproof booth in response to the ipsilaterally presented pure tone bursts of 6, 12, and 24 kHz (4-ms duration, 2-ms rise/fall time) at a rate of 21.1 stimuli/s, using an Audiology Lab system (Otoconsult, Frankfurt a. M., Germany). A calibrated loudspeaker (DT48, Beyer Dynamic, Heilbronn, Germany) accordingly presented the acoustic stimuli via a plastic cone in the outer ear canal. The ABR waveforms were thus obtained (n = 500 waves) within a time window of 10 ms. Three subdermal needle electrodes were also located at the vertex (noninverting) and under the left mastoid (inverting) and the right (ground) ear [39,40] (Fig. 3). As well, electrode impedances ranged from 1 to 3 kΩ for the electrode pairs. The sampling rate setting was 60 kHz, and the filtering of the evoked potentials was 0.3–3.0 kHz, stored for offline analysis. Besides, a custom-written MATLAB (2016a, The MathWorks Inc.) program autonomously detected the ABR characteristics based on the peak amplitude, the prominence of the ABR waves, and the time gap between every two consecutive peaks. The ABR characteristics evaluated in the present study included hearing thresholds, absolute latencies, peak amplitudes related to the I, II, III, IV, and V waveforms, the amplitude ratios of II/I, IV/I, V/I, and the BTT. The time interval between the wave I peak (viz., auditory-nerve response) and the wave valley following the wave V (namely, the wave V descending part endpoint) was further measured as the auditory BTT (Fig. 4). Likewise, the electrophysiological hearing threshold levels were automatically determined, and then validated by a neuroscientist at the 10-dB steps descending from the

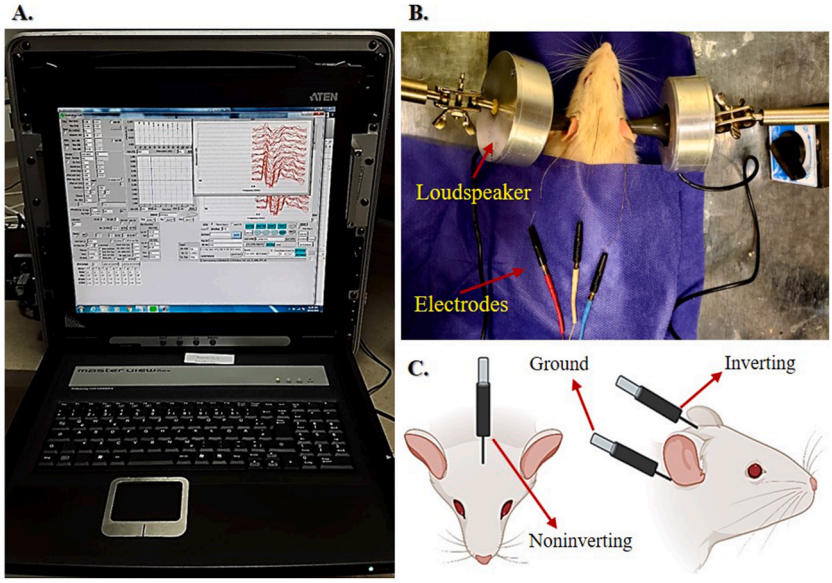

Fig. 3. Auditory Brainstem Response (ABR) Recording Setup. (A) Auditory Lab System, (B) The figure indicates how to perform the ABR test protocol in a rat. The loudspeakers and electrons are shown with arrows. (C) Three subdermal needle electrodes were placed at the vertex (non-inverting) and under the left mastoid (inverting) and the right (ground) ear.

maximum stimulus intensity of 90-dB SPL until no response was observed by tracking the repeatable and reproducible wave II.

2.6. Statistical analysis

Based on the "equation of resources" approach [41], a sample size of 8 rats was considered in each group. All statistical analyses were performed by the Statistical Package for Social Sciences (SPSS V.16; Chicago, the United States). Based on the normal distribution of the data (assessed by Kolmogorov-Smirnov) and homogeneity of variance (evaluated by Levene's test), in both groups (Chronic study group and control group), repeated-measures analysis of variance was conducted on the GPIAS the PPI, and the ABR characteristics. The Bonferroni post-hoc test was performed for multiple comparisons.

All results were presented as the mean ± standard deviation (SD). The p-value <0.05 was considered statistically significant.

3. Results

3.1. Gap pre-pulse inhibition of the acoustic startle (GPIAS) and pre-pulse inhibition (PPI) assessments

Under the baseline conditions, the discrepancies in the GPIAS and PPI mean values in percentage were not statistically significant between the control (66.21 ± 3.99 and 72.92 ± 5.27, respectively) and chronic study (67.34 ± 4.08 and 69.38 ± 4.37, respectively) groups. These results indicated that the animals in both groups had normal hearing conditions at the baseline (Fig. 5A).

Compared with the baseline, the GPIAS mean score in the chronic study group decreased drastically (27.19 ± 1.91) after 14 consecutive days of the SS injections ($p < 0.0011$). In contrast, NPPC-treated rats showed significant ($P < 0.0011$) enhancement in the GPIAS mean value (48.50 ± 6.05) compared to the 14 days. The GPIAS results thus indicated a significant suppressive effect of NPPC on SS-induced tinnitus. The PPI values in the chronic study (69.78 ± 6.82) and control (68.58 ± 6.88) groups also did not show any significant differences ($p > 0.05$) (Fig. 5B).

3.2. Differences in ABR characteristics

3.2.1. ABR threshold differences between and within chronic study and control groups

The ABR results showed significant differences in the hearing thresholds between the chronic study and control groups at the frequencies of 6 ($p < 0.001$), 12 ($p < 0.001$), and 24 kHz ($p < 0.001$) (Table 1).

A significant increase in ABR threshold was recorded after SS injection (day 14) compared to baseline in the chronic study group ($p < 0.001$). However, NPPC significantly reduced ABR thresholds (day 28) at 6 kHz ($p = 0.04$) as well as at 12 and 24 kHz ($p < 0.001$), which indicates better treatment outcomes for the higher frequency. Furthermore, baseline and day 28 stage differences were

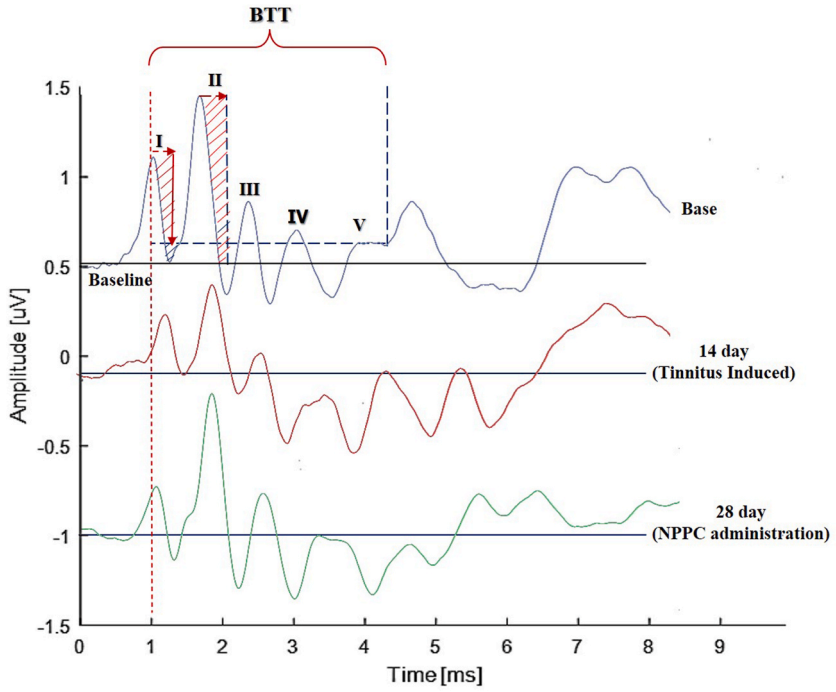

Fig. 4. An example of the baseline, post-SS (day 14), and post-NPPC (day 28) administration ABR recordings. Note the decrease in the amplitude responses compared to the baseline recordings and after the NPPC. The BTT is also considered as the interval between the peak waves I–V.

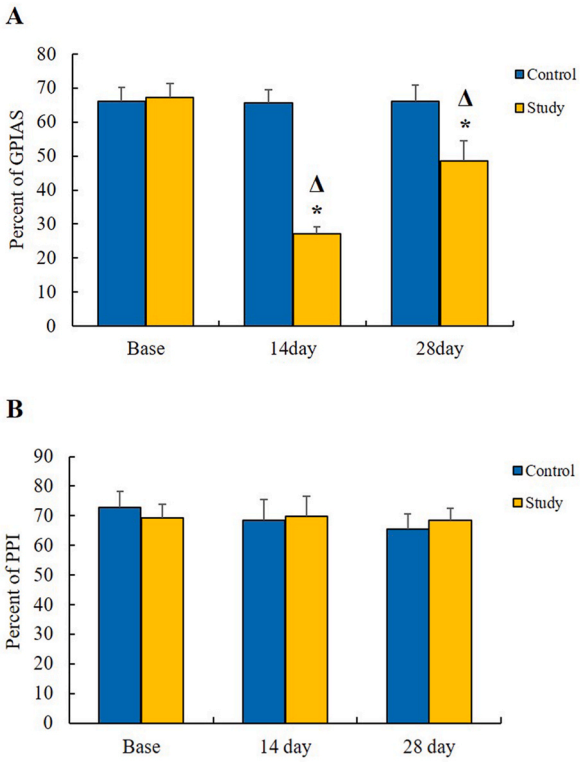

Fig. 5. GPIAS and PPI measurement results *(A)* The effects of the SS and the NPPC on the GPIAS values. The chronic study group indicated a significant decrease in the GPIAS values compared with the baseline and the control group. Asterisks (*) represent the significance between the control and chronic study groups, while the triangles (Δ) show significant value differences within each group. *(B)* The effects of the SS on the PPI values. No significant differences were seen in the PPI values between both study groups ($p > 0.05$).

insignificant at 12 and 24 kHz, suggesting a relative recovery of hearing conditions similar to the normal baseline after NPPC treatment. Post hoc analyses in the control group did not show significant differences in ABR thresholds at any of the three frequencies ($p > 0.05$ for each pair). These results indicate that NPPC has no significant effect on hearing threshold in normal rats (viz., control group). Fig. 6A shows examples of the ABR threshold waveforms from the baseline, tinnitus, and NPPC-treated rats, and Fig. 6B illustrates the summaries of the ABR threshold levels.

3.2.2. *Amplitudes of waves*

No significant difference was observed between the chronic and control study groups in all waves' amplitudes at all frequencies tested at the baseline ($p > 0.05$). However, data analysis between both groups showed a significant decrease in wave I amplitude ($p < 0.001$) and wave II amplitude ($p = 0.002$) on day 14 in the study group compared to the control group at all frequencies (Table 2).

On the 28th day, the waves I and II amplitude significantly dropped at 12 and 24 kHz ($p < 0.001$). In addition, the comparative analyses exhibited no significant differences for the amplitudes of waves III, IV, and V at the frequency of 6 kHz and waves IV and V at 24 kHz at any of the three-time stages ($p > 0.05$). Despite this, significant differences were observed between the chronic study and control groups for the ABR wave III at the frequency of 24 kHz on day 28 ($p = 0.02$), wave IV on day 28 ($p = 0.002$), and the wave V on day 14 ($p = 0.032$) at the frequency of 12 kHz.

Post hoc analysis in the chronic study group showed a significant decrease in the amplitudes of waves I and II on day 14 compared

Table 1
Mean ± SD for the ABR thresholds at three times: before (baseline), after the SS administration [day 14], and following the NPPC injection (day 28).

	Frequency KHz	Baseline [Mean ± SD]	Day 14 [Mean ± SD]	Day 28 [Mean ± SD]	*P*.value [Sig.]
Control Group	6	23.75 ± 5.17	22.50 ± 7.07	21.25 ± 6.40	0.59
	12	22.50 ± 7.07	22.50 ± 4.62	18.75 ± 8.34	0.39
	24	26.25 ± 5.17	23.75 ± 5.17	21.25 ± 3.53	0.14
Study Group	6	27.50 ± 4.62	45.00 ± 11.95	35.00 ± 5.34	<0.001
	12	26.25 ± 5.17	55.50 ± 9.25	31.25 ± 9.91	<0.001
	24	26.25 ± 5.17	55.00 ± 7.55	32.50 ± 7.07	<0.001

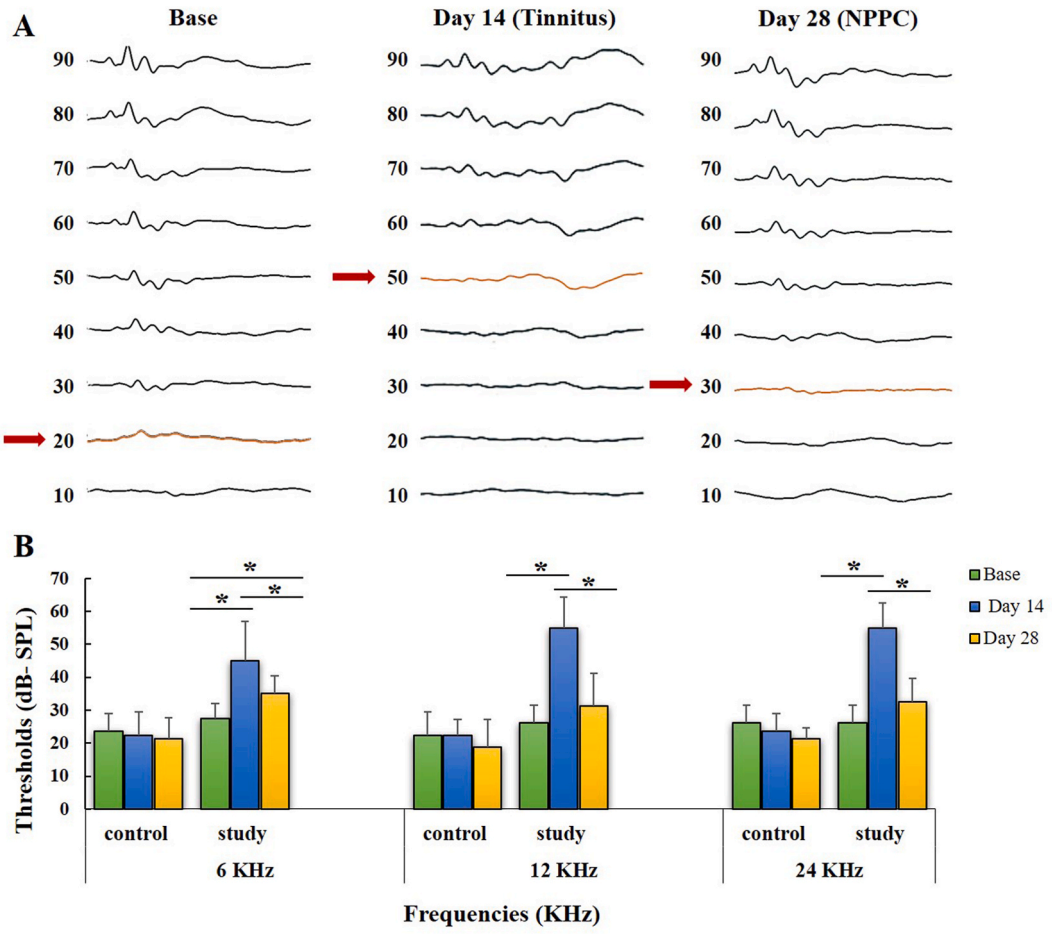

Fig. 6. The effects of the SS and the NPPC on the ABR hearing threshold shift in both study groups (A) The waveforms of the ABRs compared at the baseline, tinnitus (day 14), and NPPC administration (day 28). The arrows indicate the hearing threshold. (B) The summary of the averaged ABR thresholds at various frequencies of 6, 12, and 24 KHz. The asterisks (*) represent the pairwise comparisons within the control and chronic study groups ($p < 0.05$).

to baseline, while a significant recovery of amplitudes of waves I and II ($p < 0.001$) was observed after NPPC treatment. (namely, day 28).

The statistical results within the chronic study group also showed a significant difference in the paired comparisons of wave III at the frequency of 12 kHz between the baseline and day 14, as well as between day 14 and day 28 (Table 2, Fig. 7). No notable difference was observed in the wave IV amplitude at all frequencies and any of the three-time stages ($p > 0.05$). The exact intra-group comparisons of the wave V amplitude also showed no significant differences at the frequencies 6 and 24 kHz ($p > 0.05$). In contrast, a considerable difference was mentioned between the baseline and day 14 ($p < 0.001$), as well as between day 14 and day 28 ($p = 0.002$) at the frequency of 12 kHz. No significant differences were evident between the amplitudes of any ABR waves in the control group ($p > 0.05$).

3.2.3. Absolute latencies

Analysis of the absolute peak latencies of the chronic study group at the three-time stages indicated significant differences in the peak I latency at the frequencies of 6 and 24 kHz ($p = 0.01$ and $p = 0.03$, respectively). The chronic study group also had significant differences in the ABR wave peak III and IV latencies at 6 kHz. However, no significant differences were seen for other absolute peak latencies. Post hoc analysis also confirmed significant increases in pairwise comparisons of absolute peak latencies of wave I at 6, 12, and 24 kHz and waves III and IV at 6 kHz after SS injection. However, after NPPC treatment, a significant decrease in peak wave latencies was observed. Accordingly, as shown in Table 3, a significant recovery was evident after the NPPC treatment for the frequencies mentioned above (Fig. 8).

Table 2
The amplitude values of the ABR waves at three frequencies and three-time stages in the chronic study and control groups.

Group	Amplitude	Frequency [KHz]	Base [Mean ± SD]	Day 14 [Mean ± SD]	Day 28 [Mean ± SD]	*P*.value [Sig.]
Control	**Wave I**	6	0.84 ± 0.11	0.87 ± 0.12	0.88 ± 0.12	0.437
		12	0.83 ± 0.07	0.82 ± 0.08	0.84 ± 0.07	0.625
		24	0.85 ± 0.08	0.82 ± 0.07	0.86 ± 0.07	0.053
	Wave II	6	1.02 ± 0.08	1.01 ± 0.06	1.05 ± 0.05	0.785
		12	1.09 ± 0.08	1.08 ± 0.11	1.06 ± 0.04	0.825
		24	1.05 ± 0.08	1.02 ± 0.05	1.02 ± 0.04	0.542
	Wave III	6	0.36 ± 0.14	0.33 ± 0.14	0.34 ± 0.15	0.833
		12	0.50 ± 0.31	0.54 ± 0.13	0.51 ± 0.28	0.974
		24	0.33 ± 0.05	0.36 ± 0.04	0.36 ± 0.03	0.681
	Wave IV	6	0.33 ± 0.05	0.33 ± 0.03	0.35 ± 0.05	0.620
		12	0.33 ± 0.05	0.30 ± 0.05	0.33 ± 0.03	0.647
		24	0.33 ± 0.08	0.35 ± 0.11	0.37 ± 0.11	0.301
	Wave V	6	0.23 ± 0.09	0.25 ± 0.10	0.27 ± 0.10	0.158
		12	0.26 ± 0.06	0.25 ± 0.06	0.26 ± 0.08	0.749
		24	0.24 ± 0.06	0.24 ± 0.05	0.23 ± 0.05	0.564
Study	**Wave I**	6	0.80 ± 0.13	0.32 ± 0.09	0.61 ± 0.04	***< 0.001***
		12	0.84 ± 0.07	0.39 ± 0.06	0.58 ± 0.05	***< 0.001***
		24	0.88 ± 0.07	0.41 ± 0.04	0.57 ± 0.03	***< 0.001***
	Wave II	6	1.01 ± 0.20	0.43 ± 0.14	0.90 ± 0.25	***< 0.001***
		12	1.02 ± 0.05	0.61 ± 0.10	0.84 ± 0.15	***0.025***
		24	1.00 ± 0.05	0.53 ± 0.08	0.68 ± 0.09	***< 0.001***
	Wave III	6	0.38 ± 0.11	0.28 ± 0.12	0.44 ± 0.12	***0.014***
		12	0.55 ± 0.27	0.32 ± 0.12	0.50 ± 0.22	***0.022***
		24	0.36 ± 0.07	0.28 ± 0.10	0.28 ± 0.04	***0.040***
	Wave IV	6	0.27 ± 0.12	0.25 ± 0.21	0.32 ± 0.10	0.309
		12	0.28 ± 0.21	0.28 ± 0.20	0.20 ± 0.08	0.334
		24	0.33 ± 0.07	0.27 ± 0.10	0.30 ± 0.06	0.293
	Wave V	6	0.26 ± 0.10	0.23 ± 0.04	0.24 ± 0.08	0.490
		12	0.25 ± 0.06	0.17 ± 0.06	0.25 ± 0.06	***< 0.001***
		24	0.23 ± 0.06	0.19 ± 0.08	0.20 ± 0.05	0.106

3.2.4. Amplitude ratios

No significant difference was observed between the chronic study and control groups in the wave amplitude ratios of II/I, IV/I, and V/I at all frequencies tested at the baseline on day 28 ($p > 0.05$).

However, the same comparison on the 14th day showed significant differences in the wave amplitude ratio of IV/I at the frequency of 24 kHz ($p = 0.029$) and the wave amplitude ratio of V/I at the frequency of 6 kHz ($p < 0.001$).

Post hoc Bonferroni analysis within the chronic study group, comparing day 14 and day 28 with the baseline state, revealed significant differences for the amplitude wave ratio of II/I at the frequency of 12 kHz ($p = 0.02$ and $p = 0.033$, respectively), as well as between the baseline and the day-14 stages at the frequency of 24 kHz ($p = 0.038$).

The same analysis of the wave amplitude ratio of IV/I on days 14 and 28 compared to the baseline showed significant differences at 24 kHz ($p = 0.004$ and $p = 0.001$, respectively), and on day 28 at 6 kHz ($p = 0.014$, respectively).

The amplitude ratio of IV/I at the frequency of 12 kHz showed significant differences between the baseline and the 14th day ($p = 0.009$) as well as between day 14 and day 28 ($p = 0.044$, respectively). The amplitude ratio of V/I showed significant differences on days 14 and 28 in comparison to the baseline at 6 kHz ($p < 0.001$ and $p = 0.009$, respectively), 12 kHz ($p = 0.001$ and $p = 0.001$, respectively) and 24 kHz ($p = 0.008$ and $p = 0.004$, respectively). Also, in the comparison between days 14 and 28, a significant difference was observed in the frequency of 6 ($p < 0.001$) and 24 kHz ($p < 0.03$). Overall, all the wave amplitude ratios of II/I, IV/I, and V/I were elevated after the SS injections, but the administration of NPPCN improved the effects of salicylate. Of note, the post hoc Bonferroni analysis within the control group did not show statistically significant differences between any of the three-time stages and all frequencies ($p > 0.05$) (see Table 4) (Fig. 9).

3.2.5. Brainstem transmission time (BTT)

The time interval between the peak of wave I (the distal portion of the auditory nerve) and the endpoint of wave V descending part was considered as an auditory brain stem transmission time (BTT) (Fig. 4). The comparison of BTT between the chronic study and control groups showed statistically significant differences at three frequencies of 6, 12, and 24 kHz only on day 14 ($p = 0.02$, $p = 0.002$, and $p = 0.02$, respectively). Post hoc Bonferroni analysis within the chronic study group indicated that the mean BTT was significantly different at all frequencies between the baseline and the day-14 stages ($p = 0.04$, $p = 0.04$, and $p = 0.01$, respectively). These results revealed that chronic high doses of sodium salicylate administration cause BTT prolongation. The comparison between baseline and day 28, which was insignificant, indicated that NPPC could have positive ameliorating effects on the BTT. The post hoc Bonferroni analysis did not display any statistically significant differences between the three-time stages and the frequencies in the control group ($p > 0.05$) (Fig. 10).

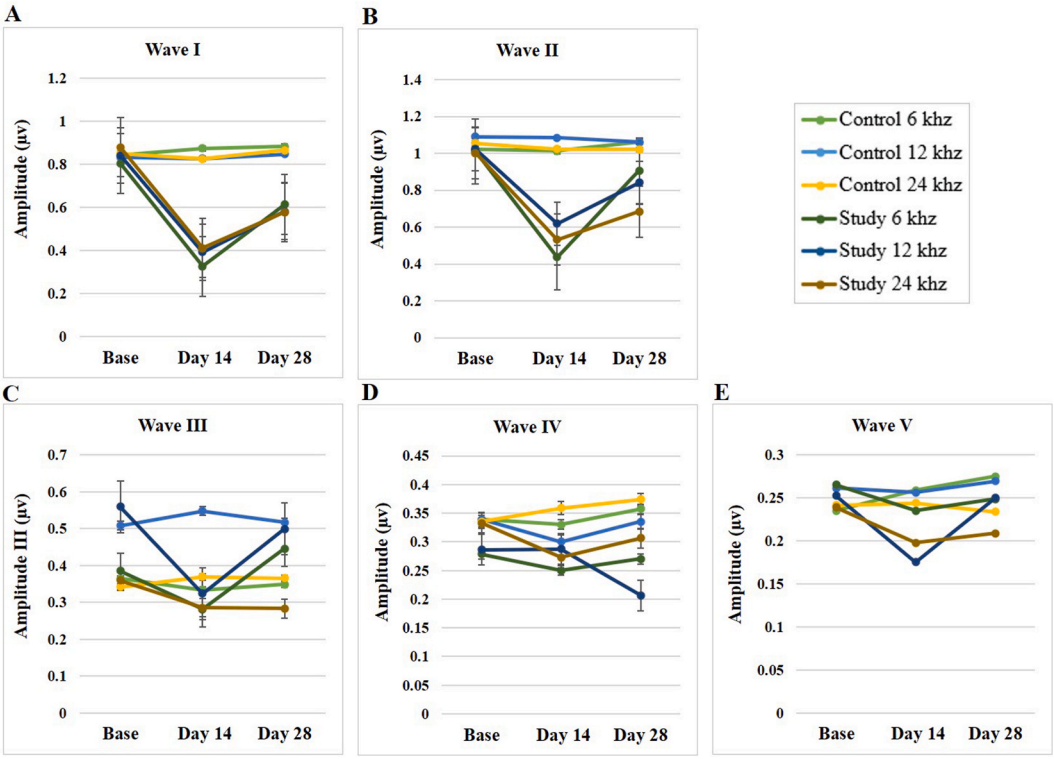

Fig. 7. The effects of the SS and the NPPC on the ABR amplitude shift in the rats. The amplitude shift were determined using 6, 12 and 24 kHz tone burst stimulations (n = 8), calculated before the SS administration (baseline), the day 14 after the SS administration (the day 14), and day 14 after the SS + NPPC administration (the day 28). *(A)* Amplitude of wave I *(B)* amplitude of wave II *(C)* amplitude of wave III *(D)* amplitude of wave IV *(E)* amplitude of wave V. The statistical significance level was considered as ($p < 0.05$).

4. Discussion

For the first time, the results of the present study showed that placental neuroprotective protein compounds (NPPC) could have a therapeutic effect against salicylate-related ototoxic effects and could suppress SS-induced tinnitus. This study describes electrophysiological changes in the underlying pathophysiology of tinnitus and subsequent improvement after NPPC administration.

Our results showed that significant changes in ABR characteristics, such as an increase in ABR threshold, decrease in amplitude, especially waves I and II, enhancement of the amplitude ratios of II/I, IV/I, and V/I, and prolongation of BTT were evident in SS-treated rats compared to the control group. Also, the behavioral evidence of tinnitus was reflected in the behavioral findings indexed by the auditory startle response after salicylate administration. As well as a significant improvement in some critical characteristics of ABR recordings as well as tinnitus suppression is evident after NPPC administration.

Tinnitus is primarily associated with sensorineural hearing loss caused by acoustic trauma, traumatic brain injury, and ototoxicity drugs. Among the list of drug-related ototoxicity, salicylate, the active ingredient of aspirin, is a non-steroidal anti-inflammatory drug that causes reversible hearing loss and tinnitus [42]. The investigations on the pathophysiological changes in SS-induced ototoxicity have proven that the central and peripheral auditory systems play critical roles in tinnitus generation [43]. Therefore, tinnitus caused by salicylate interacts with the auditory system in both the cochlea and the central auditory system. Salicylate has several independent effects on the cochlear function, which include the down-regulation of prestin, transmembrane protein in the outer hair cells, increased activity of NMDA receptors following interaction with the arachidonic acid cycle, and ultimately increased spontaneous firing rate in auditory nerve fibers [44,45].

In the central auditory system, salicylate reduces the activity of GABA and serotonin in the auditory cortex. Salicylates also increase the sensitivity of the more central parts of the auditory system to sound, which is reflected in increased startle responses, potentially causing hyperacusis. Chronic salicylate administration reduces spontaneous firing rates in the primary auditory cortex [44,45].

A hearing loss of cochlear origin, which is associated with a lowered level of activity in cochlear hair cells, may trigger the tinnitus-related neural alterations that could extend from the periphery (the inner ear) to the central auditory cortex and perhaps broader cortical and subcortical regions in the brain as shown by brain mapping studies [42,46]. This relation between cochlear hearing loss and central tinnitus is significant since recent studies have shown that in NIHL incidence of tinnitus and associated synaptic imbalance is mediated by neuroinflammation in the auditory cortex, the resolution of which may prevent tinnitus development [47]. More recently, a study using a rodent model of salicylate-induced tinnitus showed that pro-inflammatory activation of astrocytes and microglia (neuroglia) in the auditory cortex played a significant role in tinnitus development [48]. On the other hand, previous

Table 3
The absolute latency values of the ABR waves at three frequencies and three time stages in the chronic study and control groups.

Group	Absolute Latency	Frequency [KHz]	Baseline [Mean ± SD]	Day 14 [Mean ± SD]	Day 28 [Mean ± SD]	*P*.value [Sig.]
Control	**Wave I**	6	1.35 ± 0.05	1.31 ± 0.04	1.32 ± 0.08	0.485
		12	1.27 ± 0.03	1.29 ± 0.06	1.24 ± 0.06	0.566
		24	1.25 ± 0.07	1.31 ± 0.08	1.21 ± 0.09	0.098
	Wave II	6	2.05 ± 0.08	2.06 ± 0.06	2.12 ± 0.06	0.314
		12	2.00 ± 0.10	2.05 ± 0.03	2.15 ± 0.13	0.764
		24	1.97 ± 0.09	1.98 ± 0.08	2.00 ± 0.08	0.742
	Wave III	6	2.99 ± 0.22	3.06 ± 0.29	3.09 ± 0.27	0.308
		12	2.94 ± 0.22	3.02 ± 0.16	3.08 ± 0.07	0.231
		24	2.88 ± 0.23	3.00 ± 0.17	3.00 ± 0.24	0.741
	Wave IV	6	3.69 ± 0.15	3.79 ± 0.25	3.71 ± 0.25	0.937
		12	3.61 ± 0.15	3.72 ± 0.19	3.70 ± 0.20	0.778
		24	3.65 ± 0.20	3.67 ± 0.16	3.62 ± 0.11	0.990
	Wave V	6	4.62 ± 0.32	4.86 ± 0.25	4.73 ± 0.30	0.738
		12	4.66 ± 0.29	4.71 ± 0.28	4.55 ± 0.32	0.685
		24	4.50 ± 0.14	4.66 ± 0.25	4.77 ± 0.27	0.605
Study	**Wave I**	6	1.36 ± 0.05	1.50 ± 0.14	1.38 ± 0.06	***0.015***
		12	1.28 ± 0.04	1.44 ± 0.18	1.35 ± 0.08	***0.050***
		24	1.32 ± 0.02	1.43 ± 0.17	1.39 ± 0.07	***0.039***
	Wave II	6	2.12 ± 0.09	2.22 ± 0.15	2.17 ± 0.11	0.178
		12	2.05 ± 0.06	2.07 ± 0.23	2.15 ± 0.11	0.086
		24	2.11 ± 0.04	2.05 ± 0.26	2.20 ± 0.10	0.076
	Wave III	6	2.87 ± 0.12	3.42 ± 0.40	2.99 ± 0.15	***0.008***
		12	2.82 ± 0.08	3.17 ± 0.41	2.94 ± 0.16	0.065
		24	3.14 ± 0.46	2.92 ± 0.41	3.18 ± 0.47	0.396
	Wave IV	6	3.81 ± 0.20	4.84 ± 1.14	4.05 ± 0.32	0.006
		12	3.91 ± 0.47	4.43 ± 0.70	3.91 ± 0.18	0.054
		24	4.06 ± 0.48	4.40 ± 1.01	4.31 ± 0.65	0.470
	Wave V	6	5.29 ± 0.53	5.54 ± .088	5.23 ± 0.22	0.261
		12	5.27 ± 0.69	5.48 ± 0.66	5.18 ± 0.36	0.314
		24	4.99 ± 0.55	5.02 ± 0.62	5.35 ± 0.68	0.359

research has confirmed the association of salicylate toxicity with oxidative stress [49]. Human placenta extracts (HPEs) are obtained by lysing human placental tissues collected at full-term delivery. According to the data obtained from various research studies, HPEs possess anti-inflammatory, analgesic [50], antioxidant, antiapoptotic [51,52], cytoprotective and radioprotective [53], and anti-allergic properties and express hormonal activity [54], as well as stimulate proliferation and regenerative processes [55]. Based on such studies that showed the multi-potent features of HPEs, and the pathophysiology of tinnitus, as a complex medical condition with no standard treatment, we hypothesized that placenta extract might be able to reverse salicylate-induced changes. To the best of our knowledge, this is the first study that evaluated the effect of HPE in, tinnitus treatment.

The ABR data analysis indicated that the wave I amplitude decreased (representing the peripheral input loss and hidden hearing loss) and the wave amplitude ratios of V/I and IV/I elevated (denoting the increased central neuronal gain) after the long-term SS administration. The literature on the pharmacological treatment and the electrophysiological assessment of blast-induced tinnitus mainly indicates that the growth in the ABR wave amplitude ratios of V/I and IV/I might serve as a reliable parameter to identify tinnitus objectively [20,56,57]. The present study on SS-induced tinnitus, in agreement with previous research, accordingly revealed a correlation between the reduction in the ABR wave I amplitude and the enhancement in the wave amplitude ratios of IV/I and V/I, followed by compensatory plasticity changes in the central auditory pathway. Furthermore, the results of the present study provided physiological evidence that the NPPC could counteract this apparent CNS dysfunction in response to the reduced cochlear neural output. SS can traverse the blood-brain barrier [BBB] and directly act on the central auditory system [58]. In vitro, studies suggested SS probably diminishes the inhibitory post-synaptic currents [59,60], increasing spontaneous activity [61]. Besides, in vivo investigation revealed that SS may affect cochlear fast synaptic transmission through the activation of the N-methyl-D-aspartate (NMDA) glutamate receptors, which can, in turn, cause the occurrence of tinnitus [62]. Once administered systemically, SS can directly impact the functions of the auditory cortex by decreasing the inhibitory effects of the gamma-aminobutyric acid GABA-ergic neurons [60]. Moreover, another study confirmed that the cochlea-originated tinnitus and the associated synaptic imbalance had been mediated by the auditory cortex neuroinflammation, which is a resolution that might prevent tinnitus development [47]. Therefore, the underlying pathophysiology of tinnitus includes neuroinflammatory activities and synaptopathy in the auditory nervous system, which requires further investigation in this field.

The NPPC as an HPE contains diverse multifunctional proteins involved in oxidant detoxification and oxidative stress response, cytoprotection, immunity, and inflammatory response regulation, with neuroprotection, tissue regeneration, and wound healing properties [29,30]. Therefore, NPPC, through this remarkable diversity, may provide a synergistic biological function that can address different pathological aspects of tinnitus-manifesting neuro-otological disorders. Abd-Elhakim et al. (2022) recorded an increase in malondialdehyde (MDA) and a decrease in glutathione levels (GSH) and catalase activity after the administration of salicylate. Exposure to salicylate significantly induces pathological changes in cochlear and vestibular tissue and increases the expression of apoptotic factors like caspase-3 and the inflammatory indicator nuclear factor kappa (NF-κB) [63]. Several studies have revealed the

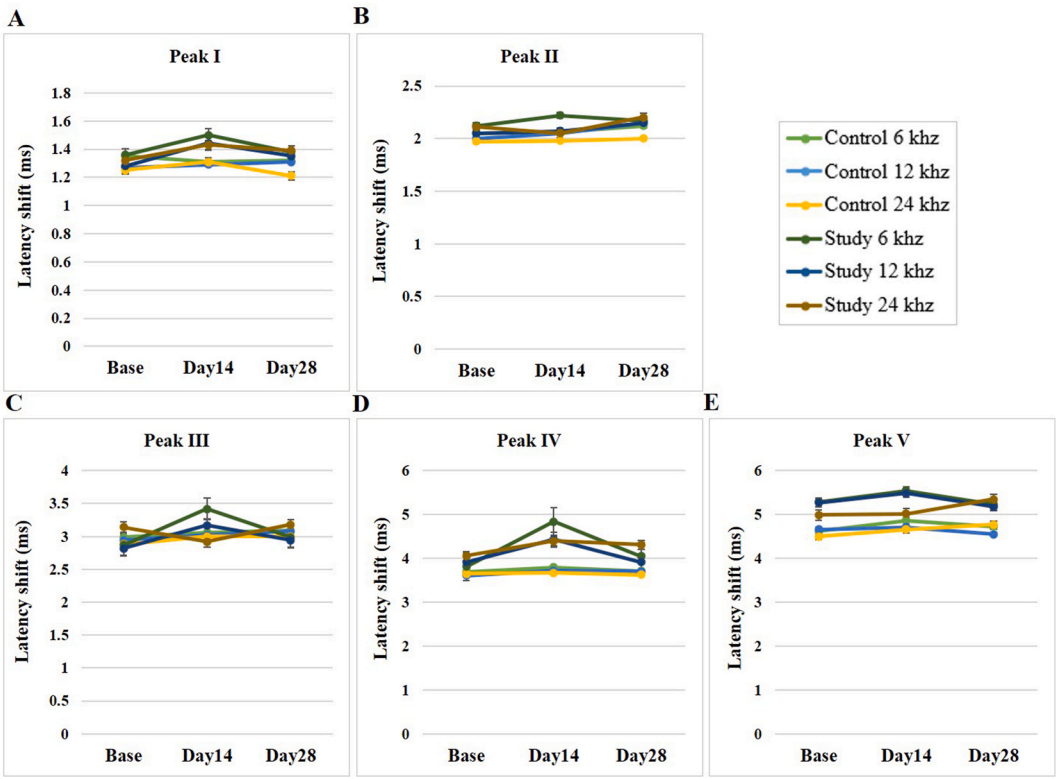

Fig. 8. The effects of the SS and the NPPC on the ABR absolute latency shift in the rats (n = 8). The absolute latency shifts were determined using 6, 12 and 24 kHz tone burst stimulations, calculated before the SS administration (baseline), after the SS administration (day 14), and the day 14 after the SS + NPPC administration (day 28). *(A)* Absolute latency shift of peak I *(B)* absolute latency shift of peak II *(C)* absolute latency shift of peak III *(D)* absolute latency shift of peak IV *(E)* absolute latency shift of peak V.

Table 4
The amplitude ratio values of the ABR waves at three frequencies and three time stages in the chronic study and control groups.

Group	Amp. Ratio	Frequency [KHz]	Base	Day 14	Day 28	*p*.value
			[Mean ± SD]	[Mean ± SD]	[Mean ± SD]	
Control	**Wave II/I**	6	1.25 ± 0.18	1.17 ± 0.19	1.21 ± 0.26	0.784
		12	1.31 ± 0.13	1.31 ± 0.17	1.25 ± 0.08	0.736
		24	1.24 ± 0.15	1.24 ± 0.15	1.18 ± 0.11	0.328
	Wave IV/I	6	0.41 ± 0.10	0.38 ± 0.07	0.41 ± 0.08	0.978
		12	0.40 ± 0.06	0.35 ± 0.06	0.39 ± 0.05	0.91
		24	0.38 ± 0.08	0.42 ± 0.11	0.42 ± 0.11	0.546
	Wave V/I	6	0.28 ± 0.14	0.29 ± 0.13	0.31 ± 0.14	0.37
		12	0.31 ± 0.07	0.31 ± 0.09	0.32 ± 0.12	0.932
		24	0.28 ± 0.07	0.29 ± 0.08	0.26 ± 0.06	0.385
Study	**Wave II/I**	6	1.27 ± 0.34	1.41 ± 0.62	1.46 ± 0.39	0.176
		12	1.22 ± 0.13	1.63 ± 0.52	1.45 ± 0.28	***0.006***
		24	1.13 ± 0.10	1.30 ± 0.23	1.18 ± 0.17	***0.033***
	Wave IV/I	6	0.34 ± 0.18	0.90 ± 0.97	0.51 ± 0.16	***0.004***
		12	0.34 ± 0.26	0.70 ± 0.50	0.35 ± 0.16	***0.014***
		24	0.37 ± 0.07	0.67 ± 0.26	0.52 ± 0.10	***< 0.001***
	Wave V/I	6	0.32 ± 0.12	0.75 ± 0.21	0.40 ± 0.14	***< 0.001***
		12	0.30 ± 0.10	0.44 ± 0.17	0.42 ± 0.09	***< 0.001***
		24	0.26 ± 0.08	0.48 ± 0.25	0.36 ± 0.11	***0.006***

anti-inflammatory and anti-oxidative activities of HPE in different models [64,65]. These studies show that HPE can decrease the level of MDA, increase GSH levels, and markedly enhance the antioxidant enzyme activities of catalase (CAT) and superoxide dismutase (SOD). Therefore, it can be said that one of the NPPC mechanisms that probably suppresses salicylate-induced tinnitus is its antioxidant effect by increasing non-enzymatic defense systems such as GSH and the activity of antioxidant enzymes such as SOD and CAT.

The results of the present study indicated the reduction of amplitudes of the ABR wave peaks I and II after the SS injection at the

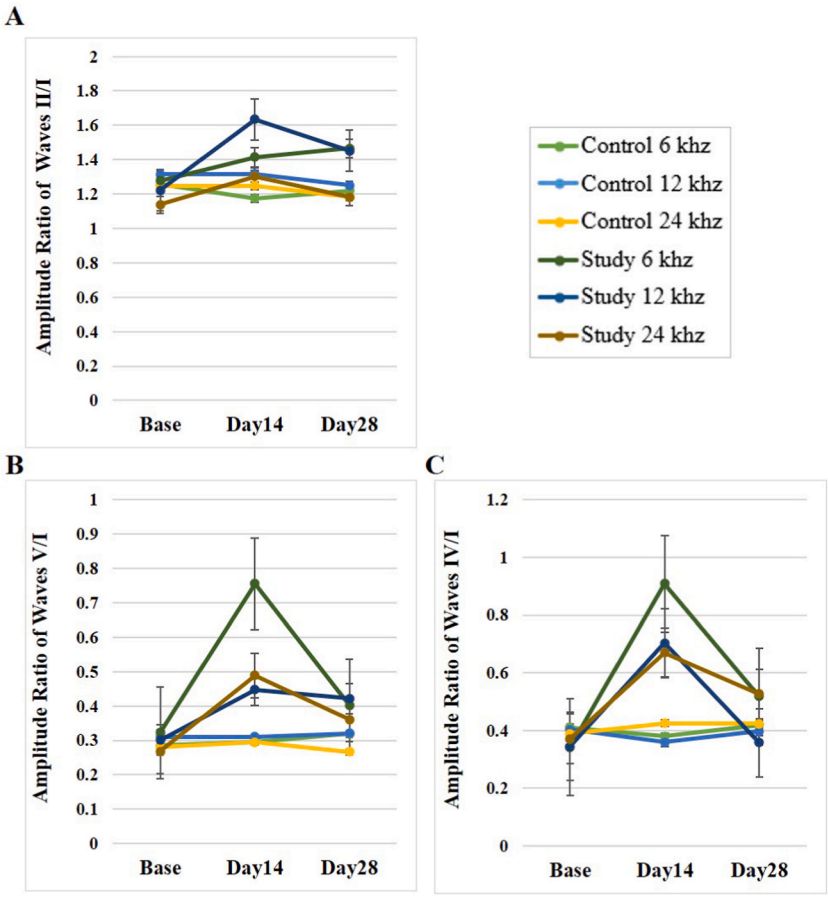

Fig. 9. The effects of the SS and the NPPC on the ABR amplitude ratios in the rats (n = 8). The amplitude ratios were determined using 6, 12 and 24 kHz tone burst stimulations, calculated before the SS administration (baseline), after the SS administration (day 14), and day 14 after the SS + NPPC administration (day 28). *(A)* Amplitude ratio of waves II/I *(B)* Amplitude ratio of waves V/I *(C)* Amplitude ratio of waves IV/I.

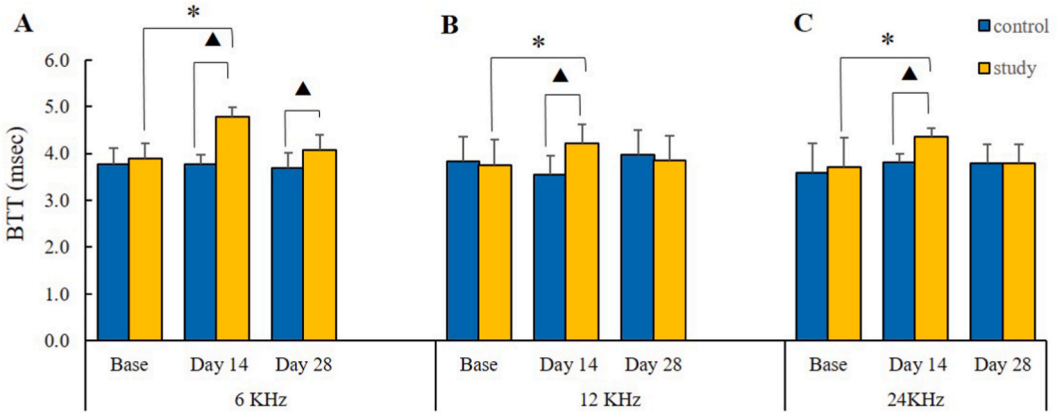

Fig. 10. BTT results. The effects of the SS and the NPPC on the BTT values. The chronic study group indicated a significant increase in the BTT values on day 14 (the SS injection) compared with the baseline, while the BTT value dropped after the NPPC administration on day 28 compared to that on day 14. *(A)* BTT of 6 KHz *(B)* BTT of 12 KHz *(C)* BTT of 24 KHz. Asterisks (*) represent the significance between the control and chronic study groups, whereas the triangles (Δ) show significant value differences within each group ($p < 0.05$).

three frequencies of 6, 12, and 24 kHz, while the ABR wave III amplitude showed a reduction at the frequency of 12 kHz, following this procedure. A previous study in mice with SS-induced tinnitus exhibited decreased ABR wave peak amplitudes and increased amplitude ratios at the frequency range of 6–24 kHz [66]. Another investigation on high-dose SS-injected rats had similarly indicated that the ABR wave peak amplitudes could be enhanced, followed by forwarding acoustic masking at high frequencies [67]. Also, the results of the present study showed that chronic administration of SS increased hearing thresholds in all frequencies, and after administration of NPPC for two weeks, ABR thresholds relatively returned to normal values. These findings are in line with numerous investigations on the animal models of noise-induced tinnitus or SS toxicity that had identified the ABR changes associated with tinnitus [18,66]. It is hypothesized that the SS-induced ototoxicity reduced cochlear output and the auditory threshold increased as a result [68]. In the present study, it was shown that along with the increase in hearing threshold, the amplitude of wave I decreased. It has been further reported that the reduced wave I amplitude and the enhanced auditory threshold might show the diminished sensory input or the synaptopathy condition of the cochlea found in tinnitus, which is consistent with the hidden hearing loss [69]. As previous studies have proposed neural alterations regarding the ABR characteristics in tinnitus [70], the BTT measurement in this study was utilized as a considerably more stable parameter to assess the tinnitus phenomenon [71] objectively. The BTT neuro-index also increased following the SS administration on day 14 at 6, 12, and 24 kHz (Fig. 10). However, this index decreased on the 28th day after the NPPC treatment, compared to the 14th day. According to these results, the NPPC might lessen the tinnitus-related activity, as confirmed by the GPIAS behavioral assessments. It can be due to the enhanced neural synchrony process in the auditory pathway and homeostasis improvement associated with the NPPC administration.

It is well known that another substance in the placenta is estrogen [72]. Estrogens have diverse neuromodulation functions that exert their neuromodulatory activities by inducing the expression of neurotrophic factors (NTs) such as glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factors (BDNF) [34]. It is widely accepted that BDNF can exert various functions in both the central and peripheral nervous systems by modulating synaptic plasticity, differentiation, neuronal survival, and the development of neurons [73]. Many studies in rat models have demonstrated that BDNF can reduce auditory thresholds, rescue spiral ganglion cells (SGCs) from degeneration, and repair damaged auditory nerve fibers [74,75]. Clinical improvement also has been found in patients with tinnitus and Sensorineural Hearing Loss (SNHL) after the treatment with NTs [76,77]. Therefore, NPPC may collectively induce other neurotrophic factors, such as BDNF, via its potential multifunctional effects, leading to improved ABR and auditory properties. This new therapy may represent a new promise for treating tinnitus and hearing disorders. Future studies need to address these mechanistic implications experimentally.

4.1. Study limitations

This animal study showed that NPPC may improve tinnitus and its associated electrophysiological characteristic but some limitations need further work to address some important questions: the potential mechanisms that contribute to tinnitus suppression need to be evaluated. As salicylate–induced ototoxicity leads to cochlear dysfunctions, assessment of cochlear structure and function after HPE (NPPC) administration can be helpful. The current study provides a novel line of evidence from an animal research perspective for more animal and clinical studies in the field of tinnitus management.

5. Conclusion

Our results revealed that human placental extract (NPPC) could alleviate the behavioral and electrophysiological changes in salicylate–induced tinnitus. The observed neuroelectrical alterations are characterized by a significantly decreased BTT and the enhanced amplitude of I and II waves and related thresholds that may indicate tinnitus reduction, manifesting as modulation of auditory deafferentation following NPPC-treated rats. The application of NPPC could improve tinnitus and exerts positive therapeutic effects on ABR characteristic. Additional research is thus needed to reveal the mechanism of action of this new potential therapeutic approach.

Author contribution statement

Saeid Mahmoudian, Zeinab Akbarnejad: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mohammad Farhadi: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ali Gorji: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Marjan Mirsalehi: Performed the experiments; Wrote the paper.

Alexander Borisovich Poletaev, Fereidoun Mahboudi, Mohammad Ebrahimi, Mohaddeseh Beiranvand, Mohaddeseh Dehghani Khaftari: Contributed reagents, materials, analysis tools or data, Wrote the paper.

Marcus Mülle, Abdoreza Asadpour: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Also, all authors agree to be accountable for all aspects of the study in ensuring that questions related to the accuracy or integrity of any part of the study are appropriately investigated and resolved.

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Data availability statement

Data included in article material/referenced in the article.

Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Mohammad Farhadi reports financial support was provided by Iran National Science Foundation. Dr. Saeid Mahmoudian reports financial support and administrative support were provided by ENT and Head and Neck Research Center, The Five Senses Health Institute, Iran University of Medical Sciences.

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