Effects of Cross-linked Hyaluronic Acid in a Rat Model of Vestibular and Cochlear Toxicity

Original Investigation

Erdal Erkoç[1], Aslı Çakır Çetin[1], Serpil Mungan Durankaya[2], Serap Çilaker Mıçılı[3], Pembe Keskinoğlu[4], Osman Yılmaz[5], Günay Kırkım[2], Enis Alpin Güneri[1]

[1]Department of Otorhinolaryngology, Dokuz Eylül University Faculty of Medicine, İzmir, Turkey
[2]Department of Audiology, Dokuz Eylül University Faculty of Health Sciences, İzmir, Turkey
[3]Department of Histology and Embryology, Dokuz Eylül University Faculty of Medicine, İzmir, Turkey
[4]Department of Biostatistics and Medical Informatics, Dokuz Eylül University Faculty of Medicine, İzmir, Turkey
[5]Department of Laboratory of Animal Science, Dokuz Eylül University Faculty of Medicine, İzmir, Turkey

Abstract

Objective: To investigate the effects of cross-linked hyaluronic acid (CLHA) in an experimental model of vestibulotoxicity and cochleotoxicity.

Methods: Twenty-eight female Wistar albino rats (200–250 g) were divided into four groups. Group A received 0.06 mL of 13.33 mg/mL gentamicin, Group B received 0.06 mL of CLHA, Group C received 0.03 mL of 26.66 mg/mL gentamicin and 0.03 mL CLHA, and Group D received 0.06 mL of 0.09% saline. All groups underwent auditory brainstem response testing at 4–32 kHz, signal-to-noise ratio of distortion product otoacoustic emission measurements at 1.5–8 kHz and vestibular tests on days 0,1,7,10. The rats were sacrificed, and their labyrinths were histologically assessed and scored.

Results: The hearing thresholds of Groups A and C were similar and significantly higher than those of the other groups at all frequencies, beginning from day 1. The vestibular and histological scores of Groups A and C were similar and significantly higher than those of the other groups from day 1. The audiological results, vestibular scores, and histological scores of Groups B and D were similar, except for a temporary middle ear effusion and hearing threshold shift in Group B. No significant deterioration was observed in the audiological, vestibular, and histological analyses of Groups B and D.

Conclusion: That both Group A and Group C similarly showed worsening audiological, vestibular, and histological tests suggests that CLHA did not alter the pharmacokinetics and histologic results of gentamicin.

Keywords: Gentamicin, hyaluronan, drug-related ototoxicity, hearing loss, audiology, vestibular function tests, animal research

ORCID IDs of the authors:

E.E. 0000-0002-4282-5321;
A.Ç.Ç. 0000-0002-2549-0494;
S.M.D. 0000-0003-4236-434X;
S.Ç.M. 0000-0002-9830-0989;
P.K. 0000-0002-3459-1828;
O.Y. 0000-0001-7817-7576;
G.K. 0000-0003-4170-5317;
E.A.G. 0000-0003-2592-0463.

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Corresponding Author:
Erdal Erkoç;
drerdalerkoc@gmail.com

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Introduction

The transtympanic administration of gentamicin is a well-known experimental model of combined vestibulotoxicity and cochleotoxicity. The dose and delivery protocol, the length of exposure, the middle ear volume, round window (RW)/oval window (OW) permeability, systemic diseases, window permeability modifiers and inner ear drug delivery systems may alter the toxicity of gentamicin (1-4). Hyaluronan (HA) is an endogenous glycosaminoglycan that structurally

participates in the various tissues of the human body, even in perilymph (5). However, it may alter the perilymphatic pharmacokinetics of an intratympanically administered drug via several mechanisms (1, 6, 7). In addition to its experimental and clinical use for inner ear drug delivery, the antibacterial, anti-adhesive, and regenerative properties of HA allow for its use in many otological applications such as middle ear packing, tympanic membrane repair, management of chronic otitis media and hearing preservation during cochlear implantation (8).

Cross-linked hyaluronic acid (CLHA) is a more stable type of HA that prevents rapid elimination of the natural liquid form (5). In previous studies, the effects of CLHA were examined only audiologically or histopathologically (9-12). This study aimed to examine the impact of CLHA on the inner ear in audiological, vestibular, and histopathological aspects in a gentamicin-induced cochleo-vestibulotoxicity model.

Methods

Animals

The study was approved by the local ethics committee (protocol date: 07/18/2018 and protocol number: 52/2018) of the Dokuz Eylül University Department of Scientific Research Projects of the Ministry of Health. Twenty-eight female Wistar albino rats weighing 200–250 g were used. They were bred and settled at a temperature of 20–25 °C with relative humidity and 12 hours of light and dark cycle in standard cages sized 50×80×100 cm. Standard pellets and tap water were supplied ad libitum. Three animals were placed in each cage.

Study Design and Intervention

This experimental study was conducted in March and April 2019. Twenty-eight rats with normal hearing and balance functions, according to the baseline audiological and vestibular assessment, were randomized into four groups to receive one of the investigational drugs intratympanically every day between 14:30 and 15:30 hours for seven days. Group A received only 0.06 mL of gentamicin at a concentration of 13.33 mg/mL (Genta, Ulagar Turkish Inc., İstanbul, Turkey), Group B received 0.06 mL of CLHA (PureRegen Gel Otol 2 mL, Bioregen Biomedical, Changzhou Co. Ltd.), Group C received 0.03 mL gentamicin at a concentration of 26.66 mg/mL and 0.03 mL CLHA, and Group D received 0.06 mL 0.09% saline. All rats were anesthetized by intraperitoneal injections (i. p.) of 50 mg/kg of ketamine hydrochloride (Ketalar 500 mg flk, Pfizer Company, USA) and 5 mg/kg of xylazine (Xylamed 5 mg/mL flk, Bimeda Animal Health Limited, Ireland) before intratympanic injections. Intratympanic injections were administered to the anteroinferior quadrant of the right tympanic membrane with a 20-gauge needle (B. Braun Venofix 20G, B. Braun Melsungen Medical Device Company, Germany) under otomicroscopy (Zeiss OPMI-1, Carl Zeiss AG, Germany), daily for a week.

Investigation of Cochlear and Vestibular Toxicity

The animals underwent vestibular and cochlear measurements on days 0, 1, 7, and 10 between 07:00 and 10:30 hours, and 11:00 and 14:30 hours, respectively. Cochlear toxicity assessment was done by measuring auditory brainstem responses (ABR) [Intelligent Hearing Systems Smart-EP l0 (lHS Corp, Miami, FL, USA)] and distortion product otoacoustic emission (DPOAE) (Otodynamics Echoport ILO-V6 Cochlear Emission Analyzer 5.61, Otodynamics, London, UK), on days 0 (before intratympanic injections), 1, 7, and 10. subdermal needle electrodes (Neuroline Subdermal, 12×0.5 mm, Ambu A/S, Malaysia) were used to record the ABRs. Tone-burst stimuli were presented at 4, 8, 16, 20, and 32 kHz, with a 1 ms rise-fall by a Blackman window envelope, in alternating polarity. Acoustic signals were recorded by bandpass filtering at 30–3000 Hz, and A/D was converted at a sampling rate of 25 kHz. The analysis time was set at 10 ms and the artifact rejection level at 31.00. ABR waves were obtained using 1000 stimuli presented at a rate of 37.1/s. DPOAE was measured using a neonatal probe. The f2/f1 ratio was maintained at 1.22. The levels of the stimulus were L1 (65 dB SPL) for f1 frequency and L2 (55 dB SPL) for f2 frequency. The baseline hearing condition of rats was measured with DPOAE and the signal-to-noise ratio was recorded at seven frequencies between 1500 and 8000 Hz. The vestibular test battery included tail-hanging, air-righting reflex, and swimming (13). The tail hanging test evaluated unilateral contraction and rotational movements of the rats attached to their tails with plastic plasters and suspended from a 50 cm high table for two minutes. The air-righting test measured the rats' ability to correct their posture when held on the back at a height of 30 cm for two minutes and subsequently dropped on a surface of soft textiles and foam. The swimming test was performed in a 30 cm diameter and 50 cm height cylindrical-shaped polyethylene pool filled with 30 cm tap water at a constant temperature of 37 °C. The deterioration in swimming quality and turning around the tail axis was observed for two minutes. After the swimming test, the grooming skill (ability to dry itself) was also observed for two minutes. Each animal was tested separately, and each test had a 10-minute interval during which the rats were allowed to rest in their cages. The rats were dried under a warm light source for 30 minutes after the swimming test. Behavioral tests were recorded using a camera (Eken 9HR 4 K Action Camera, Eken Electronics Ltd. Shenzhen, China) attached to a 15 cm height tripod located 30 cm away from the test field. The vestibular tests of the rats were videotaped and scored. Vestibular dysfunction score (VDS) refers to the sum of all vestibular assessment items (Table 1) (13, 14).

Table 1. Vestibular dysfunction score

	Score			
Variables	**0**	**1**	**2**	**3**
1- Tail hanging	No visible sign	Faint presence of the sign	Clear evidence of the sign	Maximum expression of the sign
2- Tail hanging tumbling	No visible sign	Faint presence of the sign	Clear evidence of the sign	Maximum expression of the sign
3- Air-righting reflex	Perfect preparation of the two front paws before reaching the ground	Mild impairment of the two front paws before reaching the ground	Severe impairment of the two front paws before reaching the ground	Absence of preparation for landing
4- Swimming turn to tail axis	No visible sign	Faint presence of the sign	Clear evidence of the sign	Maximum expression of the sign
5- Swimming quality	Normal swimming	Irregular swimming	Immobile floating	Underwater tumbling
6- Grooming after swimming	No visible sign	Faint presence of the sign	Clear evidence of the sign	Maximum expression of the sign

Explanatory table of the vestibular influence measurement method. In this table, the effect levels of drug-administered rats in 6 different categories were scored between 0 and 3. Rats with no effect got 0 points and those who were maximally affected got 3 points

Sacrification of Rats and Histopathological Examination

On the 10th day, after the final vestibular and cochlear evaluation, the rats were sacrificed using intracardiac ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (5 mg/kg). The tympanic bulla was resected. Tissues were fixed in 10% formalin solution for three days. Decalcification was accomplished using ethylenediaminetetraacetic acid solution at room temperature for a month. The decalcified tissues were embedded in paraffin blocks and 5 µm sections were taken with a rotary microtome (Leica, RM 2255, USA). The sections were stained with hematoxylin and eosin (H&E) (ab245880, Abcam, England) and evaluated under light microscopy (BH2, Olympos, Japan). Apoptosis was evaluated by TUNEL assay (the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) and activated Caspase-3 immunohistochemical staining. Chromatin concentration, nuclear fragmentation, shrinking of the cytoplasm, and formation of apoptotic bodies were considered as indicators of apoptosis (15).

The TUNEL Assay

The TUNEL assay was used to demonstrate DNA fragmentation. After deparaffinization, the sections were kept at first in Proteinase K (64220, Abcam, England) at 37 ˚C for 10 minutes and then in terminal deoxyribonucleotide transferase at 37 ˚C for 60 minutes. After converter peroxidase (POD) was applied, the sections were stained with diaminobenzidine (DAB, 1718096, Roche, Mannheim, Germany) and covered with entellan (Merck, Darmstadt, Germany) and background staining with Mayer hematoxylin (15).

Activated Caspase-3 Immunohistochemistry

After deparaffinization, the sections were heated in a microwave oven with 10 mM citrate buffer for 10 minutes. Tissues were limited with DakoPen (PAP pen, Dako Denmark APS, Denmark). Then, to inhibit endogenous tissue POD, 3% hydrogen peroxide was applied to the sections for five minutes. The sections, which were washed three times for five minutes with phosphate buffer solution, incubated with a blocking solution at room temperature for an hour and then incubated with anti-caspase 3 activated form (MAB10753, 1:100 dilution Sigma, Germany) at 4 ˚C without washing. A biotinylated secondary antibody (Histostain-Plus Broad Spectrum 85-Invıtrogene, Carlsbad, CA) and streptavidin were applied for 30 minutes, respectively. DAB was used to make the reaction visible. Ground staining was performed with Mayer hematoxylin. Sections, of which dehydration was carried out in graded alcohols, were covered with Entellan (15). The basilar membrane, spiral ganglion, cochlear nerve, stria vascularis, outer hairy cells, utricular and saccular macula, and apoptosis were evaluated and scored. The scores of these items represented the histological score (HS) (Table 2).

Statistical Analysis

The experimental outcomes were the ABR thresholds at individual frequencies, VDS, and HS. The Kolmogorov–Smirnov test was used for normal distribution analysis. Parametric quantitative variables were defined as mean ± standard deviation. For non-parametric quantitative variables, the median, minimum and maximum values were also calculated. Statistical analyses were done using SPSS version 22.0 (IBM Corp., Armonk, NY, USA) at a 95% confidence interval. One-way analysis of variance (ANOVA) and post-hoc Tukey-Kramer tests were used to compare the parametric quantitative variables between independent groups. The Kruskal–Wallis test and post-hoc Dunn–Bonferroni adjustment were used to compare the non-parametric quantitative variables between independent groups. The Friedman test and post-hoc Dunn–Bonferroni adjustment were used to assess the difference between non-

Table 2. Histomorphological score

	Score			
Variables	**0**	**1**	**2**	**3**
1- Spiral ganglion	No injury	Slight injury	Moderate injury	Severe injury, unrecognizable tissue morphology
2- Cochlear nerve	No injury	Slight injury	Moderate injury	Severe injury, unrecognizable tissue morphology
3- Stria vascularis	Absence of shrinkage of the intermediate cells	Slight shrinkage of the intermediate cells	Moderate shrinkage of the intermediate cells	Severe shrinkage of the intermediate cells
4- Outer hair cell (OHC)	Three OHCs with intact nuclei	Two OHCs with intact nuclei	One OHC with intact nuclei	No OHCs with intact nuclei
5- Utricular and Saccular macula	No injury	Slight injury	Moderate injury	Severe injury, unrecognizable tissue morphology
6- Apoptosis	No apoptotic cells	1–5 apoptotic cells	6–10 apoptotic cells	>10 apoptotic cells

Explanatory table of histological exposure measurement method. In this table, after the temporal dissection of drug-administered rats, cells in five different areas were examined for histological changes. While maximum exposure was 3 points, the areas that were not affected at all were scored as 0 points

parametric repeated measures of each group; $p<0.05$ was considered the minimum level of significance.

Blinding

Randomization and intratympanic injections were performed by a non-blinded investigator. Blinded authors performed intraperitoneal anesthesia, ABR and DPOAE recordings, vestibular assessment and scoring, sacrification, histological evaluation, and statistical analyses.

Results

None of the animals died and/or were excluded from the study during the experiment. Thus, the statistical analyses included all 28 animals.

Groups A and C showed significant cochleotoxicity at 4, 16, 32 kHz ABR frequencies in the first 24 hours ($p<0.05$). In Group A, the hearing thresholds were found to be progressively impaired and the worst hearing threshold was reached on the 10th day. Compared to the control group (Group D), significant differences were identified at all frequencies on days 1, 7 and 10. No significant differences were found between Groups A and C regarding the hearing status at any frequency measured on days 1, 7, and 10 ($p>0.05$) (Table 3).

In Group C, the hearing thresholds increased significantly at the 24th hour at 4 kHz (Mean ± SD; 26.43±2.4 dB $p<0.05$). Hearing deterioration was statistically significant at all frequencies on days 7 and 10 ($p<0.05$).

There were no significant differences between Groups B and D regarding the hearing status on days 1, 7 and 10 ($p>0.05$). In Group B, however, temporary increase in hearing thresholds was observed on day 7, although no differences were found in the statistical analysis.

VDSs at the 24th hour of Group A (Mean ± SD; 5.43±1.3) and Group C (Mean ± SD; 3.57±1.4) were significantly higher compared to Group B (Mean ± SD; 0.00) and Group D (Mean ± SD; 0.57±1.1) ($p<0.05$).

The statistical analysis of the VDSs between Groups A and C showed non-significant differences ($p>0.05$). In both groups, vestibular damage was identified at the maximum level on the 10th day.

In Groups A and C, microscopic damage in the organ of corti, the stria vascularis, the spiral ganglion, the cochlear nerve, the outer hairy cells, the support cells, and the basilar membrane were recorded. Neuronal degeneration and sparsely located neurons were detected in the spiral ganglion. Group C scored the highest in the histological results. However, the pair-wise analysis did not show any significant differences between Group A (Mean ± SD; 13.14±1.2) and Group C (Mean ± SD; 14.29±1.1) ($p>0.05$). To sum up, common and combined cochleovestibular damage was evident in Groups A and C, without a tendency for isolated involvement of either the cochlea or the vestibule.

According to the pair-wise analysis, on the other hand, the total histological damage scores of Group B (Mean ± SD; 2.71±1.7) and Group D (Mean ± SD; 1.71±1.6) were very low and not significantly different from each other ($p>0.05$).

The results of frequency specific ABR thresholds, VDS, and HS of the groups on days 0, 1, 7, and 10, and the intra- and inter-group differences are summarized in Table 3 and shown in Figures 1, 2, and 3.

Table 3. Results of outcome measures of the groups on days 0, 1, 7, and 10; and the significance values of the statistical analyses between groups and measurements

			Day 0	Day 1	Day 7	Day 10	Friedman p
ABR 4 kH	Group A (gentamicin, n=7)	Mean ± SD	22.14±9.1	25.71±3.5	40.71±1.9	51.43±18	**0.00***
		Median (min, max)	20 (10–40)	25 (20–30)	40 (40–45)	40 (40–85)	
	Group B (CLHA, n=7)	Mean ± SD	19.29±3.5	22.14±2.7	32.71±4.6	22.86±2.7	**0.00***
		Median (min, max)	20 (15–25)	20 (20–25)	35 (25–39)	25 (20–25)	
	Group C (gentamicin + CLHA, n=7)	Mean ± SD	22.14±2.7	26.43±2.4	52.86±6.4	40±10	**0.00***
		Median (min, max)	20 (20–25)	25 (25–30)	50 (45–65)	35 (30–60)	
	Group D (saline, n=7)	Mean ± SD	19.29±1.9	20±0	23.57±4.8	20±2.9	0.05
		Median (min, max)	20 (15–20)	20 (20–20)	20 (20–30)	20 (15–25)	
	Kruskal–Wallis p		0.26	0.01	0.00	0.00	
ABR 8 kHz	Group A (gentamicin, n=7)	Mean ± SD	21.43±5.6	30.71±3.5	55.71±16	72.14±20	**0.00***
		Median (min, max)	25 (10–25)	30 (25–35)	60 (35–80)	65 (55–105)	
	Group B (CLHA, n=7)	Mean ± SD	21.43±3.8	26.43±7.5	39.86±8.2	36.43±3.8	**0.00***
		Median (min, max)	20 (15–25)	25(15–40)	39(30–55)	35 (30–40)	
	Group C (gentamicin + CLHA, n=7)	Mean ± SD	22.14±2.7	28.57±5.6	58.57±7.5	51.43±14	**0.00***
		Median (min, max)	20 (20–25)	30 (20–35)	55 (55–75)	45 (35–70)	
	Group D (saline, n=7)	Mean ± SD	17.86±2.7	22.14±3.9	25±4.1	17.86±5.7	**0.02***
		Median (min, max)	20 (15–20)	25 (15–25)	25 (20–30)	15 (15–30)	
	Kruskal–Wallis p		0.10	0.02	0.00	0.00	
ABR 16 kHz	Group A (gentamicin, n=7)	Mean ± SD	12.86±2.7	23.57±4.8	61.43±17	68.57±18	**0.00***
		Median (min, max)	15 (10–15)	20 (20–30)	55 (45–90)	65 (45–95)	
	Group B (CLHA, n=7)	Mean ± SD	14.29±3.5	19.29±3.5	33.57±6.3	30±5.8	**0.00***
		Median (min, max)	15 (10–20)	20 (15–25)	30 (25–40)	30 (25–40)	
	Group C (gentamicin + CLHA, n=7)	Mean ± SD	14.29±1.9	20±0	62.86±9.9	47.14±13	**0.00***
		Median (min, max)	15 (10–15)	20 (20–20)	60 (45–75)	45 (30–65)	
	Group D (saline, n=7)	Mean ± SD	16.43±3.8	15±4.1	19.29±3.5	17.86±5.7	0.38
		Median (min, max)	15 (10–20)	15 (10–20)	20 (15–25)	15 (15–30)	
	Kruskal–Wallis p		0.22	**0.00***	**0.00***	**0.00***	
ABR 20 kHz	Group A (gentamicin, n=7)	Mean ± SD	15.71±1.9	22.14±6.4	62.86±11	69.29±12	**0.00***
		Median (min, max)	15 (15–20)	20 (15–30)	65 (45–75)	65 (55–85)	
	Group B (CLHA, n=7)	Mean ± SD	18.57±2.4	18.57±2.4	37.14±9.5	35±5	**0.00***
		Median (min, max)	20 (15–20)	20 (15–20)	35 (25–55)	35 (30–40)	
	Group C (gentamicin + CLHA, n=7)	Mean ± SD	16.43±2.4	21.43±3.8	61.43±10	61.43±9.9	**0.00***
		Median (min, max)	15 (15–20)	20 (15–25)	65 (45–75)	60 (45–75)	
	Group D (saline, n=7)	Mean ± SD	16.43±3.8	17.14±2.7	20.71±3.5	20±7.6	0.47
		Median (min, max)	15 (10–20)	15 (15–20)	20 (15–25)	15 (15–35)	
	Kruskal–Wallis p		0.22	0.13	**0.00***	**0.00***	
ABR 32 kHz	Group A (gentamicin, n=7)	Mean ± SD	17.86±2.7	28.57±4.8	78.57±7.5	82.86±7	**0.00***
		Median (min, max)	20 (15–20)	25 (25–35)	80 (65–90)	85 (75–95)	
	Group B (CLHA, n=7)	Mean ± SD	21.43±3.8	24.29±4.5	45.71±10	42.86±4.9	**0.00***
		Median (min, max)	20 (15–25)	25 (20–30)	50 (35–55)	45 (35–50)	
	Group C (gentamicin + CLHA, n=7)	Mean ± SD	20±4.1	24.29±3.5	61.43±9.9	61.43±9.9	**0.00***
		Median (min, max)	20 (15–25)	25 (20–30)	60 (45–75)	60 (45–75)	
	Group D (saline, n=7)	Mean ± SD	19.29±1.9	18.57±2.4	25±2.9	22.86±6.4	**0.01***
		Median (min, max)	20 (15–20)	20 (15–20)	25 (20–30)	20 (15–35)	
	Kruskal–Wallis p		0.25	**0.00***	**0.00***	**0.00***	

Table 3. Continued

			Day 0	Day 1	Day 7	Day 10	Friedman p
	Group A (gentamicin, n=7)	Mean ± SD	0±0	5.43±1.3	11.29±1.7	14.86±2.4	**0.00***
		Median (min, max)	0 (0–0)	6 (4–7)	12 (9–13)	16 (11–17)	
	Group B (CLHA, n=7)	Mean ± SD	0±0	0.00	1.57±1	1.43±1.3	**0.00***
		Median (min, max)	0 (0–0)	0 (0–0)	2 (0–3)	1 (0–3)	
	Group C (gentamicin + CLHA, n=7)	Mean ± SD	0±0	3.57±1.4	9.71±1.6	10.71±3.3	**0.00***
		Median (min, max)	0 (0–0)	3 (2–5)	9 (8–13)	9 (9–18)	
	Group D (saline, n=7)	Mean ± SD	0±0	0.57±1.1	0.86±1	1.14±1.5	0.18
		Median (min, max)	0 (0–0)	0 (0–3)	1 (0–2)	1 (0–4)	
	Kruskal–Wallis p		1.00	**0.00***	**0.00***	**0.00***	
HS	Group A (gentamicin, n=7)	Mean ± SD	13.14±1.2		**ANOVA p**	Post-hoc comparison and p	
		Median (min, max)	13 (12–15)			Group A-B	**0.00***
	Group B (CLHA, n=7)	Mean ± SD	2.71±1.7			Group A-C	0.89
		Median (min, max)	3 (1–5)		0.00*	Group B-C	**0.00***
	Group C (gentamicin + CLHA, n=7)	Mean ± SD	14.29±1.1			Group A-D	**0.00***
		Median (min, max)	13 (13–16)			Group C-D	**0.00***
	Group D (saline, n=7)	Mean ± SD	1.71±1.6				
		Median (min, max)	1 (0–5)			Group B-D	1.00

Statistical analysis of audiological, vestibular and histological effects. In this table, there are analyzes showing whether the audiological (ABR: 4, 8, 16, 20, 32 kHz)

ABR: Auditory brainstem response, kHz: KiloHertz, VDS: Vestibular dysfunction score, HS: Histological damage score, CLHA: Cross-linked hyaluronic acid, n: Number of animals, SD: Standard deviation, Min: Minimum, Max: Maximum

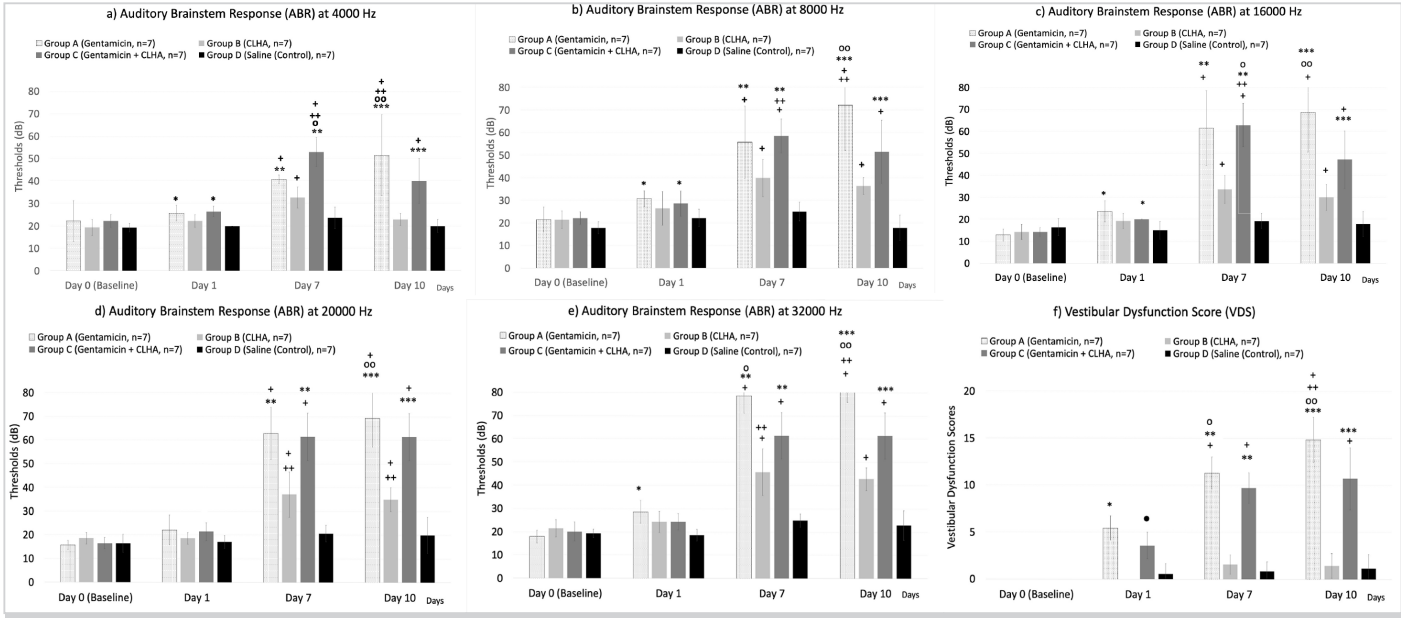

Figure 1. Frequency-specific auditory brainstem responses (ABR) and vestibular dysfunction scores (VDS) of animals (n=28) regarding groups. a) ABR at 4000 Hz, b) ABR at 8000 Hz, c) ABR at 16000 Hz, d) ABR at 20000 Hz, e) ABR at 32000 Hz, f) VDS

Frequency specific ABR and VDS scores on days 0, 1, 7 and 10.

a) ABR at 4000 Hz, b) ABR at 8000 Hz, c) ABR at 16000 Hz, d) ABR at 20000 Hz, e) ABR at 32000 Hz, f) VDS scores

*: Compared to Group saline on day 1, **: Compared to Group saline on day 7, ***: Compared to Group saline on day 10, o: Compared to Group CLHA on day 7, oo: Compared to Group CLHA on day 10, +: Compared to day 0, ++: Compared to day 1, •: Compared to day 0

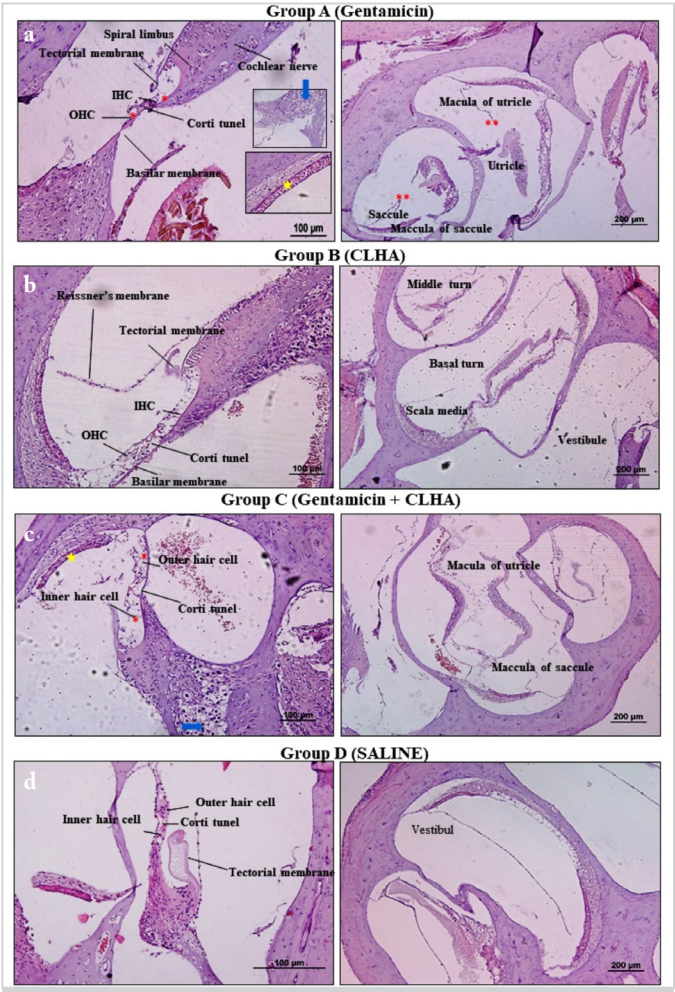

Figure 2. Hematoxylin and eosin stained histological sections. a) Group A (gentamicin) 100 µm–200 µm, b) Group B (CLHA) 100 µm–200 µm, c) Group C (gentamicin + CLHA) 100 µm–200 µm, d) Group D (Saline) 100 µm–200 µm. In Group A, the inner (IHC) and outer hair cell (OHC) loss is very evident (*). The damage in the spiral ganglion, spiral ligament, and basilar membrane, as well as in the utricle and the saccule is significant (**). Intense pycnosis and vacuolization are observed in the stria vascularis (yellow star) and the spiral ligament (blue arrow). Supporting cell loss is evident in the basilar membrane (mm: micrometers). In Group B, the organ of Corti, IHC, OHC, supporting cells and basilar membrane are almost normal. In Group C, cellular vacuolization is observed in cochlear and vestibular sections. The vacuolization and pycnosis in the spiral limbus and the spiral ganglion and the damage in the basilar membrane, supporting cells, OHC and IHC significant. In Group D, the normal histological view of all structures

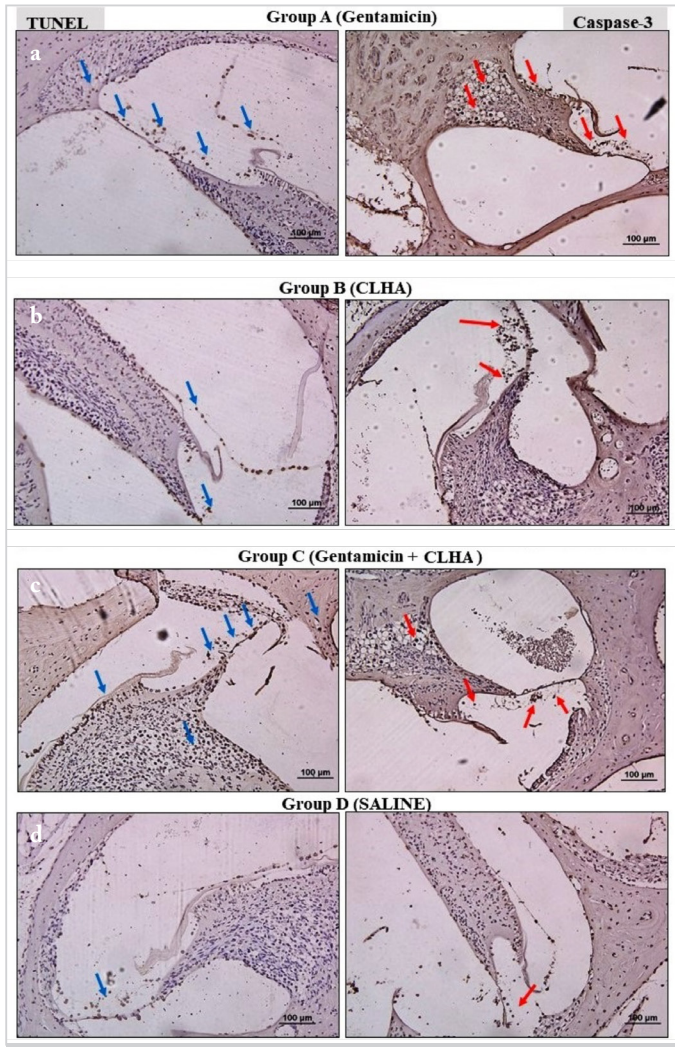

Figure 3. Active caspase-3 and TUNEL histological sections. a) Group A (gentamicin) 100 µm–200 µm, b) Group B (CLHA) 100 µm–200 µm, c) Group C (gentamicin + CLHA) 100 µm–200 µm, d) Group D (saline) 100 µm–200 µm. Representative histochemical TUNEL and caspase-3 immunostaining micrographs (Blue arrows: TUNEL positive cells, Red arrows: Caspase-3 positive cells). In Group A, red arrows indicate areas of TUNEL and activated caspase-3 positive cells. Cells marked by caspase-3, the last step of the apoptotic pathway in active caspase-3 sections, are shown by a red arrow. Mitochondrial stress factor, cytochrome C is visible. In TUNEL sections, deoxyribonucleotide ends exposed after apoptosis turned into a substrate that could not be solved at the endpoints by using terminal deoxyribonucleotide transferase and made visible by reverse methyl staining

Discussion

The transfer pathway of gentamicin from the middle ear to the inner ear remains obscure. Anatomical differences and systemic diseases affect the permeability of the RW and OW (16). According to cadaveric studies, RW and OW blockages were present in 22% and 18% of the population, respectively (17). Another study revealed that RW translucency was not available in 5% of cases and was very low in 13% of cases (18). From the perspective of gentamicin, experimental studies demonstrated that the OW could be dominating its transport (19). However, a more recent study, in contrast to other studies, suggested that most of the gentamicin (57%) intratympanically administered to the ear passed through the RW (1). Furthermore, it has been reported that when intratympanically administered, 66% of gentamicin flowed within the first 24 hours. In contrast, the remaining drug slowly diffused on the following days and were eliminated from the middle ear within 48 hours (20, 21).

Considering this controversy, we aimed to inject a volume that was adequate to completely fill the middle ear.

According to previous studies, when HA is combined with intratympanic drugs, it prolongs drug release, increases the drug's contact with the RW and OW and its diffusion into the inner ear. For instance, combined administration of HA and gentamicin has been reported to extend the release and increase the efficacy of intratympanic gentamicin and boost the perilymph concentration of intratympanic dexamethasone (5, 21). It should also be noted that its molecular structure, such as the presence and ratio of the cross-linked design, plays an essential role in its mode of action (22). It was also observed that gentamicin release kinetics varied according to the dispersion rate in a hybrid material when combined with HA (23).

In our study, gentamicin showed its preliminary cochleo-vestibulotoxic effects in the first 24 hours and continued its action in the following days, in contrast to the results of a study that reported the initiation of cochleotoxicity 5–6 weeks after the application (24). Furthermore, the emergence time of vestibular positive findings was observed to be earlier in our study, in contrast to a previous study which reported that the signs of vestibulotoxicity were observed on the 7th day following gentamicin administration (25).

The literature provides contradictory views regarding the cochleotoxicity of gentamicin. Sheppard et al. (26) stated that there were no findings suggesting that intratympanic gentamicin was vestibular selective. In another study, vestibular toxicity was achieved by intratympanic gentamicin without significant hearing loss; however, histological evidence revealed cellular toxicity in both the cochlea and the vestibule and no statistically significant difference was found between them (27). Güneri et al. (28) reported deterioration in vestibular behavioral tests with combined intratympanic gentamicin and gentamicin-dexamethasone and supported their findings by histological evidence of apoptosis.

The ABR thresholds of Group C worsened similarly to the Group A according to our results, which was also quite different from the previous studies that documented increased risk of hearing loss with sustained gentamicin release from an HA + gentamicin hydrogel compared to gentamicin alone (29). Our audiological findings were also supported by VDS and HS. However, as we used CLHA in our methodology, which is a novel experimental method, we believe that the use of different titrations and administration methods of HA may underlie this discrepancy.

On the other hand, there were no statistically significant differences between Group B and Group D regarding hearing thresholds on days 1, 7, and 10. These results are compatible with previous findings (30). The similarity of the VDS and HS results between these two groups supported the audiological findings as well. However, slight and transient threshold deterioration from day to day was observed at all frequencies in the Group B, which was statistically significant only in intragroup analyses (Table 3). Indeed, this situation was thought to occur due to the cross-linked structure of HA, which caused middle ear effusion (detected by otomicroscopic examinations) as a minor and transient adverse event while enhancing the stabilization of the HA (2, 3, 8).

We agree that, as we did not measure the absorption time, half-life, and middle ear concentration of gentamicin and CLHA in our experimental model, it may not be entirely convenient or reliable to make a clear inference from our results. This should be highlighted as a major limitation of this study. However, as a strength of our study, we utilized caspase-3 and TUNEL assays to reveal accurate histological results since H&E staining may confuse the definitive diagnosis of apoptosis as it only provides information about the morphological changes that may also occur with mechanical trauma (15, 25). Nevertheless, since we did not find any significant differences in audiological, vestibular, and histological parameters between groups A and C, and groups C and D, we assume that CLHA has no long-lasting effects on the cochlea and vestibule, other than causing temporary middle ear effusion.

Conclusion

This study evaluated the cochlear and vestibular effects of intratympanic gentamicin, gentamicin + CLHA, and CLHA alone and compared them with those of the control subjects. We believe that the novel use of gentamicin + CLHA and assessment of cochleo-vestibolotoxicity in all aspects, including both audiological and vestibular test battery, H&E staining, and apoptosis markers make our study a notable contribution to the literature. Despite the previous reports that have focused on the potential impact of HA on drug pharmacokinetics, which may alter the final efficacy or safety of the drug, we did not find any significant and permanent effect of CLHA, whether alone or in combination with gentamicin, on both the cochlea and the vestibule. We plan to elucidate the exact mechanisms underlying our results by further well-designed pharmacodynamic and pharmacokinetic studies.

Ethics Committee Approval: This research was approved by the Dokuz Eylül University Local Ethics Committee (protocol date: 07/18/2018 and protocol number: 52/2018). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed Consent: Experimental animal study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: E.E., Concept: A.Ç.Ç., S.M.D., G.K., Design: S.Ç.M., O.Y., E.A.G., Data Collection and/or Processing: E.E., S.M.D., S.Ç.M.,

Analysis and/or Interpretation: P.K., Literature Search: E.E., A.Ç.Ç., Writing: E.E., E.A.G.

Conflict of Interest: The authors have no conflicts of interest to declare.

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Main Points

- This is the only study in the literature that monitors cellular changes in both cochlear and vestibular levels and measures behavioral balance tests and audiological tests.
- While hyaluronic acid has been tried many times in other fields for 'prolonged drug release', its combined application with gentamicin in the inner ear is rare.
- Hyaluronic acid production technologies have been increasing in recent years. The combination produced by cross-linking technology hyaluronic acid and gentamicin is also unique in this context.
- Most of the studies in the literature advocate the benefits of hyaluronic acid and its cross-linked versions. However, in our study, neither a long-term drug release effect of hyaluronic acid nor a benefit for regeneration effect in toxicity in the inner ear was observed.

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