RESEARCH ARTICLE
DOI: 10.26650/Tr-ENT.2022.1216068
The Turkish Journal of Ear Nose and Throat 2022;32(4):115-120

Investigation of Ototoxicity of Intratympanic 5-fluorouracil in Rats

Meltem Demirdağ Çevikkan[1], Asım Aslan[1], Elgin Türköz Uluer[2], Damla Demirkaya Taş[2]

[1]Manisa Celal Bayar University, Faculty of Medicine, Department of Otolaryngology, Head and Neck Surgery, Manisa, Turkiye
[2]Manisa Celal Bayar University, Faculty of Medicine, Department of Histology and Embryology, Manisa, Turkiye

ORCID ID: M.D.Ç. 0000-0003-2742-0125; A.A. 0000-0003-4081-233X; E.T.U. 0000-0002-6597-6923; D.D.T. 0000-0002-6471-7671

Citation: Demirdag Cevikkan M, Aslan A, Turkoz Uluer E, Demirkaya Tas D. Investigation of ototoxicity of intratympanic 5-fluorouracil in rats. Tr-ENT 2022;32(4):115-120. https://doi.org/10.26650/Tr-ENT.2022.1216068

ABSTRACT

Objective: Although 5-fluorouracil is beneficial in treating cholesteatoma in people, it is unknown if this drug harms the inner ear. We are planning to contribute to the literature by investigating the effects of the intratympanic administration of 5-fluorouracil solution form to the middle ear on the inner ear in rats.

Materials and Methods: The study was conducted on 11 Wistar albino male rats: a positive control group was treated with amikacin, a study group was treated with 5-FU, and a negative control group received no treatment. One week after intratympanic drug administration, the rats were sacrificed, and ototoxicity was histopathologically examined by light microscopy and TUNEL (**T**erminal deoxynucleotidyl transferase-mediated d**U**TP **N**ick **E**nd **L**abeling) method.

Results: There was a significant difference between the two groups treated with amikacin and 5-fluorouracil in terms of apoptosis ($p<0.05$). The difference in stria vascularis thicknesses was significant between the amikacin group and the 5-fluorouracil group and the negative control group ($p<0.05$).

Conclusion: The intratympanic administration of 5-fluorouracil to rats did not have any ototoxic effects, according to the results of the histological analysis that looked at apoptosis.

Keywords: 5-fluorouracil, ototoxicity, intratympanic injection, animal model

INTRODUCTION

Cholesteatoma is a histopathologically benign but clinically aggressive and destructive tumor of the middle ear. Today, surgery is the known treatment of cholesteatoma (1). The surgical technique of choice is open or closed mastoidectomy depending on the location and extent of the cholesteatoma. Both surgical techniques pose a risk of morbidity. Moreover, a second surgery may be required with both techniques as residual and recurrent cholesteatoma may develop depending on the course of the disease (2). The residue and recurrence rates of cholesteatoma were shown to be greater in individuals treated with the closed approach compared to those treated with the open technique, particularly when performed by less skilled surgeons (3). To effectively combat the condition, a therapeutic approach that lowers the postoperative recurrence of cholesteatoma will be very helpful. Studies show that topical application of 5-fluorouracil (5-FU), a chemotherapeutic agent, is effective in the treatment of cholesteatoma (4, 5). In addition to disrupting DNA and RNA synthesis, 5-FU causes cell cycle arrest and apoptosis by increasing the expression of p53, a tumor suppressor gene. Considering the theories regarding cholesteatoma development, it can be understood that it is effective in the treatment of cholesteatoma (6). Recent studies have shown that the systemic absorption of topically applied 5-FU is <2%, with no evidence of any vestibular and cochlear damage in humans even with systemic use (7). Ototoxicity has been investigated by applying the cream form of 5-FU to the external auditory canal of guinea pigs, and it has been found that application to the external auditory canal is safe in terms of damage to the inner ear (8). However, there is no data on the toxicity of the intratympanic 5-FU solution form in

Corresponding Author: Meltem Demirdağ Çevikkan **E-mail:** drmeltem_8888@hotmail.com

Submitted: 07.12.2022 • **Revision Requested:** 23.12.2022 • **Last Revision Received:** 03.01.2023 • **Accepted:** 05.01.2023 • **Published Online:** 17.01.2023

the literature. It is crucial to understand whether or not 5-FU given intratympanically is ototoxic before using it to treat cholesteatomas both intraoperatively and postoperatively. The original value of the study is that it aimed to investigate the potential ototoxic effects of intratympanic administration of 5-FU solution form to the middle ear in rats.

MATERIALS AND METHODS

Rats

The approval for the study was obtained from the Ethics Committee for Animal Experimentation (Date: 21.05.2019, no: 77.637.435). In line with the Guide for the Care and Use of Laboratory Animals, animal rights were protected in our study. In this investigation, a total of 11 healthy male rats weighing between 300 and 400 grams (0.66 and 0.88 pounds) were employed. The rats were kept in cages in the same room, with 12 hours of light and 12 hours of darkness, and an average temperature of 21°C. They had access to food and water. Care was taken to keep the background noise level below 50 dB. The study was conducted in the Laboratory of Experimental Animals in accordance with the principles of Helsinki, and a histopathological examination was performed in the Laboratory of Histology and Embryology.

Anesthesia and Drug Administration

General anesthesia was provided with a mixture of 10 mg/kg xylazine HCl and 100 mg/kg ketamine HCl. The rats were first divided into 2 groups: 25 mg amikacin/rat (Amikacin sulfate, 500 mg/2 ml vial, Zentiva) was administered to 4 rats in the amikacin group as a positive control. In the 5-FU group, 5 mg 5-FU/rat (Fluorouracil, 1000mg/20 ml vial, Kocak) was administered to 7 rats. Both drugs were administered via intratympanic injection at a dose of 0.10 ml to the inferior quadrant of the right eardrum using a 27-gauge needle. The same anesthetic dose was intracardially injected into the amikacin and 5-FU groups one week after the drug administration, and then the rats were sacrificed, and their cochlea was dissected (Figure 1). The left cochleas of 3 rats in the 5-FU group, which was not given any drug, were included in the study as the third group (negative control).

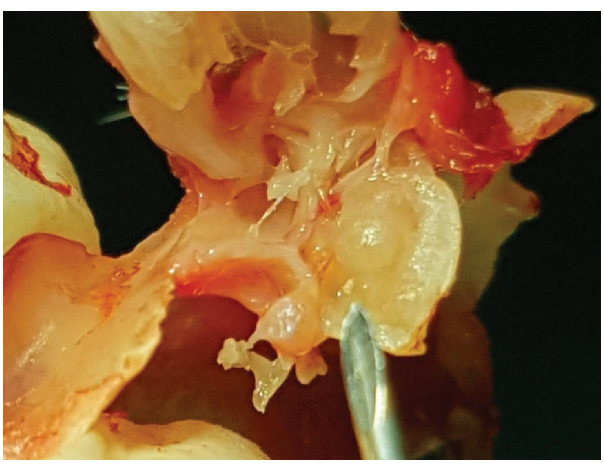

Figure 1: The rat cochlea

Histopathological Examination

Rat cochleas were fixed in a 10% formalin solution for 24 hours. After 2 weeks of decalcification in 0.1M EDTA (ethylene diamine tetra-acetic acid) solution, the tissues were rapidly dehydrated with a series of increasing concentrations of alcohol. The routine paraffin follow-up procedure was completed after the dehydrated tissues were embedded in paraffin and made transparent with xylene. From paraffin blocks, five micron-thick sections were sliced, deparaffinized, rehydrated, stained with H&S (Hematoxylin-Eosin), and examined using an Olympus BX-40 light microscope. TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling) staining was performed to determine apoptotic cell death.

With Hematoxylin-Eosin (H&S) staining, cochlear edema, cytoplasmic changes, stria vascularis thickness, and signs of inflammation as well as edema, cytoplasmic changes, lipid accumulation, vacuolization, signs of inflammation in the spiral limbus, and edema, nucleus pycnosis, increased acidophilia, satellite cell changes, and signs of inflammation in the spiral ganglion were evaluated and graded as 0- No histopathological change, 1-Minimal change, 2-Moderate change, 3-Severe change. Semi-quantitative histopathological examination was performed according to the number of degenerated cells; 0: No histopathological change, 1-5: Minimal change, 6-10: Moderate change, >10: Severe change. Apoptotic cells in the stria vascularis, spiral limbus, and spiral ganglion were counted and graded at 40x magnification as **0**: No apoptotic cells, **1**: 1-5 apoptotic cells, **2**: 6-10 apoptotic cells, and **3**: more than 10 apoptotic cells.

Statistical Analysis

Using power analysis, the sample size for each group was determined. The study used a minimum of 7 experimental animals due to a power of 80%, an effect size of d=1.40, and a significance level of 0.05. IBM SPSS Statistics v15.0 software was used for statistical analysis. Since the number of experimental animals was <30 in the study, nonparametric tests were used. The Chi-square test was used for the comparison of categorical data, and Fisher's exact test was applied when the frequency determined in the 4-cell evaluation was <5. Kruskal wallis test and Mann-Whitney U test were used for intergroup evaluation of stria vascularis thickness. Bonferroni corrections were performed for multiple comparisons. The significance level was established as α=0.05.

RESULTS

Evaluation of histological changes: The spiral ganglion and spiral limbus were the most severely affected structures, and the overall cochlear structure was impaired in the Amikacin group, according to the analysis of H&S-stained preparations. There was no significant difference between the 5-FU group and the amikacin group in terms of edema and cytoplasmic changes in the stria vascularis ($p>0.05$), and no statistical evaluation was made since no inflammation was observed in both group (Table 1). Stria vascularis thickness (µm) was compared in pairs between the amikacin group, the 5-FU group, and the negative control group; the 5-FU group (median 17.30; min-max 15.03-

21.57) had a lower stria vascularis thickness than the amikacin group (median 22.56; min-max 18.85-26.04) and a higher stria vascularis thickness than the negative control group (median 13.31; min-max 12.83-13.70), with a statistically significant difference between the groups ($p<0.05$) (Table 1).

The difference in lipid accumulation and vacuolization seen in the spiral limbus between the 5-FU group and the amikacin group was not statistically significant ($p>0.05$), according to the semi-quantitative histopathological grading presented in Table.1. While spiral limbus edema was minimal in the 5-FU group, it was minimal (25%) and moderate (75%) in the amikacin group. The 5-FU group had minimal cytoplasmic changes (86%) and no change (14%), while the entire amikacin group had moderate changes. While no spiral limbus inflammation was observed in the entire 5-FU group, the amikacin group had moderate (50%), minimal (25%) spiral limbus inflammation, and no change (25%), with statistically less spiral limbus inflammation in the 5-FU group ($p<0.05$).

Spiral ganglion edema was minimal (57%) and moderate (43%) in the 5-FU group, while it was moderate (50%) and severe (50%) in the amikacin group, with statistically less spiral ganglion edema in the 5-FU group ($p<0.05$). There was no statistically significant difference between the 5-FU and amikacin groups in terms of nucleus pycnosis and acidophilia increases in the spiral ganglion ($p>0.05$) (Tablo.1). Satellite changes in the spiral ganglion were moderate (25%) and severe (75%) in the amikacin group, while no change was observed in the 5-FU group ($p<0.05$). While the signs of spiral ganglion inflammation were minimal (75%) and moderate (25%) in the amikacin group, no sign of spiral ganglion inflammation was noted in the 5-FU group ($p<0.05$) (Figure 2,3,4).

Evaluation of apoptotic cells: The comparison of the amikacin group with the 5-FU group showed a lower number of apoptotic cells in the stria vascularis, spiral limbus, and spiral ganglion in the 5-FU group, with a statistically significant difference ($p<0.05$) (Figure 5). In addition, there was no statistically significant difference in the apoptosis grading of the stria vascularis, spiral limbus, and spiral ganglion in the 5-FU group compared to the negative control group ($p>0.05$) (Table 2).

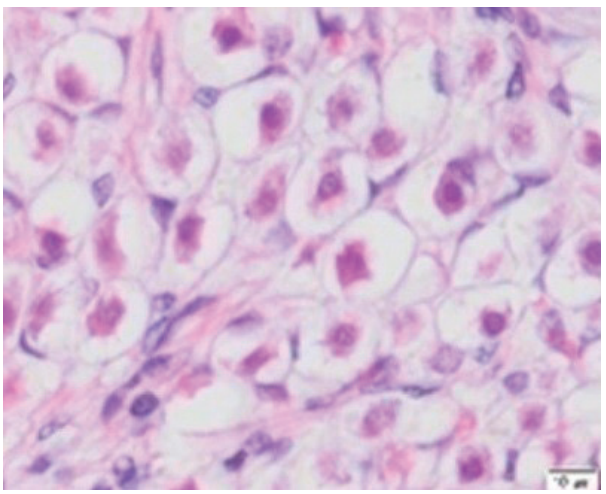

Figure 2: Hematoxylin-Eosinstaining (x400, bar= 10 µm). Spiral ganglion-The 5-FU group

Table 1: Grading of histopathological changes in the groups

		The Amikacin group				The 5-Fluorouracil group						The Negative group			
	Rat no:	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**1**	**2**	**3**
Stria vaskularis	Edema	2	1	3	0	0	1	1	0	2	1	1	1	0	0
	Cytoplasmic Changes	2	1	3	0	0	0	0	0	0	0	0	1	0	0
	Signs of inflammation	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Thickness (µm)	21.20	18.85	26.04	23.93	16.12	17.30	1623	15.03	21.57	19.02	20.23	13.31	13.70	12.83
Spiral limbus	Edema	2	2	2	1	1	1	1	1	1	1	1	0	0	0
	Cytoplasmic changes	2	2	2	2	1	0	1	1	1	1	1	0	0	0
	Lipid accumulation	0	1	0	0	1	0	1	1	0	0	0	0	0	0
	Vacuolization	2	1	0	0	0	0	0	0	0	0	0	0	0	0
	Signs of inflammation	0	2	1	2	0	0	0	0	0	0	0	0	0	0
Spiral ganglion	Edema	2	2	3	3	1	2	1	1	1	2	2	0	1	0
	Nucleus pycnosis	1	0	0	2	1	1	0	1	0	1	1	0	1	0
	Increased Acidophili	3	1	1	3	0	2	1	1	1	1	1	0	1	0
	Satellite cell changes	2	3	3	3	0	0	0	0	0	0	0	0	1	0
	Signs of inflammation	1	2	1	1	0	0	0	0	0	0	0	0	0	0

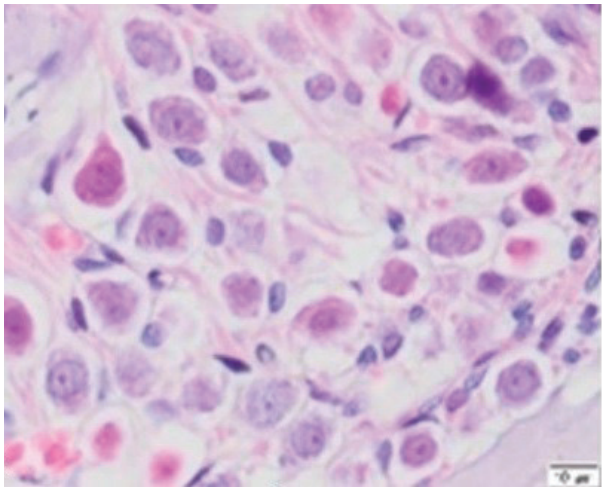

Figure 3: Hematoxylin-Eosinstaining (x400, bar= 10 µm). Spiral ganglion – The Negatif group

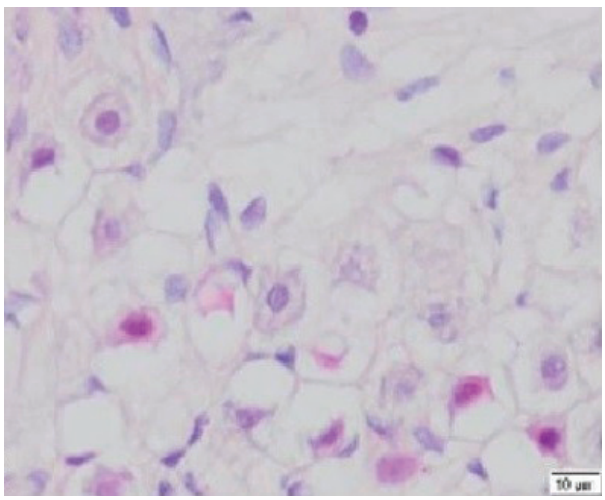

Figure 4: Hematoxylin-Eosinstaining (x400, bar= 10 µm). Spiral ganglion – The Amikasin group

(In amikacin group compared to the other two groups, there is an increase in pycnosis and edema in the nucleus and a deterioration in satellite cell distribution around the ganglion cell.)

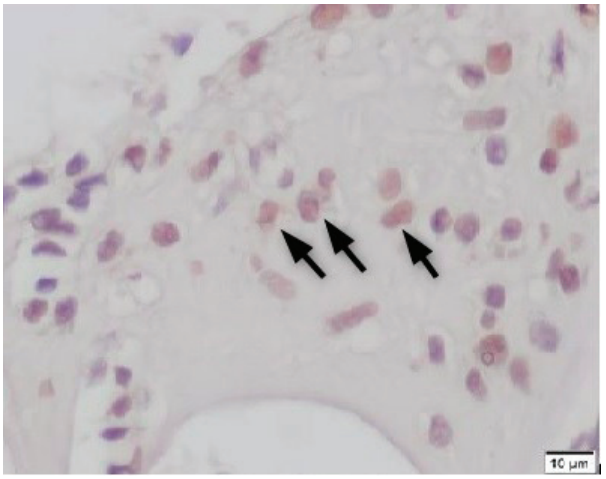

Figure 5: TUNEL method (bar= 10 µm). Spiral limbus – The Amikasin group

(Black arrows -TUNEL positiveBrown apoptotic cells with nucleus pushed aside)

DISCUSSION

Our study demonstrated no ototoxic effect of intratympanic administration in 5-FU to rats in the histopathological examination performed by evaluating apoptosis. While there is no universally accepted medical treatment for cholesteatoma, surgery is the known treatment. Studies on both humans and animals have demonstrated the effectiveness of topical 5-FU in the management of cholesteatoma (4, 8, 9). Considering that residual and recurrent cholesteatoma may develop depending on the surgical technique, the location and extent of the cholesteatoma, and the surgeon's experience, we think that intratympanic 5-FU may be a complementary treatment (2).

Our study aimed to investigate the middle ear, where cholesteatoma is most common. The intratympanic technique, which is currently used for the treatment of many diseases and is very practical and applicable, was chosen to access the middle ear. Ototoxicity was considered to be a limiting factor for intratympanic 5-FU administration. Therefore, rat cochlea

Table 2: Distribution of apoptosis in the groups

	Apoptotic cell number	The Amikacin group (n=4)	The 5-Fluorouracil group (n=7)	The Negative group (n=3)
Stria Vascularis p=0.009	**0**	-	1 (14.3%)	2 (66.7%)
	1-5	1 (25%)	6 (85.7%)	1 (33.3%)
	6-10	3 (75%)	-	-
	>10	-	-	-
Spiral Limbus P=0.020	**0**	-	-	1 (33.3%)
	1-5	-	4 (57.1%)	2 (66.7%)
	6-10	1 (25%)	3 (42.9%)	-
	>10	3 (75%)	-	-
Spiral Ganglion P=0.004	**0**	-	-	-
	1-5	-	2 (28.6%)	3 (100%)
	6-10	-	4 (57.1%)	-
	>10	4 (100%)	1 (14.3%)	-

was examined to investigate drug ototoxicity, considering intratympanic pharmacokinetics (10). 5-FU is a water-soluble chemotherapeutic agent. We believe that this feature may affect both its passage through the round window as well as its absorption by the middle ear mucosa and its residence time in the tissue. Additionally, the drug's molecular weight may have an impact on ototoxicity. Studies have shown that one of the excipients in the cream form of 5-FU, propylene glycol, lowers the endocochlear potential and induces ototoxicity. However, it is unclear whether the ototoxicity brought on by the cream form is caused by an increase in molecular weight or by propylene glycol (11, 12).

In their study on guinea pigs, Iwanaga et al. applied the cream form of 5-FU only to the external auditory canal of the first group and to the external auditory canal and the middle ear of the second group with myringotomy (5). They did not find any change in the stria vascularis thickness by light microscopy in both groups, concluding that 5-FU did not cause degeneration in the inner ear. The results of our study showed that stria vascular thickness was higher in the 5-FU group than in the negative control group and lower than in the amikacin group ($p<0.05$). However, considering apoptosis, 5-FU was similarly found to not cause degeneration in the inner ear. Iwanaga et al. connected the study's findings that the cream form of the drug might result in ototoxicity by increasing molecular weight to the group receiving drugs with myringotomy's poor electrophysiological measurements (5).

Chemotherapeutic agents usually cause ototoxicity via apoptosis (13). Examination by the TUNEL method gives an idea about apoptotic cell detection and ototoxicity. Electrophysiological tests could not be used in our study due to technical deficiencies. However, we think that more comprehensive results can be obtained by supporting histopathological findings with electrophysiological tests (7). In addition, we think that it would be useful to examine ototoxicity biochemically (14).

In our study, 5-FU was administered at a dose of 7.5-10 mg/rat, considering the maximum bulla volume of the rat. In intratympanic administration of 5-FU, it can be thought that middle ear volume limits the maximum administrable dose of the drug. However, we believe that 5-FU, which is known to have no ototoxic effect even in systemic administration, can be administered via intratympanic injection at repeated doses to increase its efficacy.

In ototoxicity, symptoms may arise immediately after drug administration or may develop within days or weeks (15). In our study, ototoxicity was assessed one week following 5-FU and amikacin administration. We think that prolonging the research duration and giving the medicine in multiple doses will lead to more accurate results for ototoxicity. Considering the side effects of 5-FU, the most serious complication observed with the cream form is chronic ulceration of the skin. Smith reported chronic ulceration in humans with the application of topical 5-FU cream to patients undergoing open surgery (4). There is no information on whether the 5-FU solution form causes pain or ulceration in the middle ear mucosa after intratympanic administration.

The histopathological examination performed by evaluating apoptosis revealed no ototoxic effect of the intratympanic administration of 5-fluorouracil in rats. The current findings imply that topical 5-FU application may be preferred as an adjunct to surgical treatment for early-stage cholesteatomas to prevent residual and recurrent cholesteatomas or to stop the progression of cholesteatoma in patients who cannot undergo the procedure because of underlying diseases. However, there is a need for controlled randomized studies supported by the electrophysiological technique to examine the efficacy of intratympanic 5-FU in both experimentally induced cholesteatoma and cholesteatoma patients.

Acknowledgements: The authors would like to thank the people who works at the experimental animals laboratory.

Ethics Committee Approval: This study was approved by the Laboratory Animals Local Ethics Committee of Manisa Celal Bayar University (Date: 21.05.2022, No: 77.637.435).

Informed Consent: Written informed consent was obtained.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- A.A.; Data Acquisition- M.D.Ç, A.A., E.T.U; Data Analysis/Interpretation- M.D.Ç., D.D.T.; Drafting Manuscript- M.D.Ç, A.A.; Critical Revision of Manuscript- A.A., E.T.U.; Final Approval and Accountability- M.D.Ç., A.A.; Material or Technical Support- E.T.U., D.D.T.; Supervision- A.A., E.T.U.

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

Financial Disclosure: This study was supported by Scientific Research Projects Coordination Unit of Manisa Celal Bayar University (grant number 109-2009).

REFERENCES

1. Olszewska E, Chodynicki S, Chyczewski L. Apoptosis in the pathogenesis of cholesteatoma in adults. Eur Arch Otorhinolaryngol 2006; 263:409-13.
2. Sheehy JL, Brackmann DE, Graham MD. Cholesteatoma Surgery: Residual and Recurrent Disease. Ann Otol Rhinol Laryngol 1977;86(4):451-62
3. Tomlin J, Chang D, McCutcheon B, and Harris J. Surgical technique and recurrence in cholesteatoma: a meta-analysis. Audiol Neurotol 2013;18(3):135-42.
4. Smith MF. The topikal use of 5-fluorouracil in the ear in the management of cholesteatoma and excessive mucous secretion. Laryngoscope 1985;95(10):1202-3.
5. Iwanaga T, Tanaka F, Tsukasaki N, Terakado M, Kaieda S, Takasaki K, et al. Does topical application of 5-fluorouracil ointment influence inner ear function? Otolaryngol Head Neck Surg 2006:134;961-5.
6. Ceilley RI. Mechanisms of action of topical 5-fluorouracil: Review and implications for the treatment of dermatological disorders. J Dermatolog Treat 2010;23(2):83-9.

7. Erlanger M, Martz G, Ott F, Storck H, Rieder J, Kessler S. Cutaneous absorption and urinary excretion of 5-fluorouracil ointment applicated in an ointment to healthy and diseased human skin. Dermatologica 1970;140 (suppl I):7-14.
8. Takahashi H, Funabiki K, Hasebe S, Yamamoto TF, Kaieda S, Iwanaga T, et al. Clinical efficacy of 5-fluorouracil (5-FU) topical cream for treatment of cholesteatoma. Auris Nasus Larynx 2005:32(4);353-7.
9. Sala DT. Topical applications of 5-fluorouracil in the medical treatment of cholesteatoma of the middle ear. Ear Nose Throat J 1994;73(6):412-4.
10. Salt AN, Plontke SK. Principles of local drug delivery to the inner ear. Audiol Neurootol 2009;14:350-60.
11. Morizono T, Paparella MM, Juhn SK. Ototoxicity of propylene glycol in experimental animals. Am J Otolaryngol 1980;1(5):393-9.
12. Wright CG, Bird LL, Meyerhoff WL. Effect of 5-fluorouracil in cholesteatoma development in an animal model. Am J Otolaryngol 1991;12(3):133-8.
13. Elmore S. Apoptosis: A Review of Programmed Cell Death. Toxicol Pathol 2007;35(4):495-516.
14. Salcan İ, Dilber M, Bayram R, Süleyman E, Yazıcı GN, Çoban A, Süleyman H. Effect of Rutin on Gentamicin-Induced Ototoxicity in Rats: A Biochemical and Histopathological Examination. ENT-Updates 2021;11(1):8-13.
15. Ganesan P, Schmiedge J, Manchaiah V, Swapna S, Dhandayutham S, Kothandaraman P P. Ototoxicity: A Challenge in Diagnosis and Treatment. J Audiol Otol 2018;22(2):59-68.