The Journal of International Advanced Otology

Int Adv Otol 2014; 10(2): 128-33 • DOI:10.5152/iao.2014.72

Original Article

Does Short Term Usage of Fresh Pomegranate Juice (FPJ) Protect Cochlear Hair Cells after Cisplatin-Based Chemo-Irradiation?

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OBJECTIVE: The aim of this study was to investigate the protective effect of short-term usage of fresh pomegranate juice (FPJ) on ototoxicity after cisplatin-based chemo-irradiation

MATERIALS and METHODS: The study was carried out on 56 adult Wistar Albino rats, which were divided into 8 groups (n: 7 for each group). The first group was accepted as the sham control group. However, rats in the seven experimental groups were treated with FPJ; cisplatin; irradiation; irradiation plus FPJ; cisplatin plus FPJ; both cisplatin and irradiation; and combined use of cisplatin, irradiation, and FPJ, respectively. Ototoxicity was evaluated by Distortion Product Otoacoustic Emissions (DPOA), histopathology, and paracochlear protein carbonyl content.

RESULTS: The results of the study showed that cisplatin, irradiation, and both cisplatin and irradiation treatments affected hair cells dramatically. However, the results of cisplatin plus FPJ and combined use of cisplatin, irradiation, and FPJ indicated that FPJ plays an important role in protecting hair cells in the inner ear. Additionally, the findings of signal-noise ratio and protein carbonyl values also supported the results stated above.

CONCLUSION: Fresh pomegranate juice treatment can be a supportive agent to reduce hair cell injury in the inner ear of patient treated by cisplatin, radiotherapy, or cisplatin-based chemo-irradiation. However, more performance is necessary for further studies, especially on long-term treatment with FPJ.

KEY WORDS: Pomegranate, irradiation, cisplatin-based chemo-irradiation, hair cells of inner ear, ototoxicity, oxidative stress, protein oxidation

INTRODUCTION

As it is known, there are several treatments that are used for the eradication of head and neck tumors, including surgery, chemotherapy, irradiation, or chemo-irradiation. Surgery is aggressive and often limits the quality of life or loss of vital organs, such as the larynx. Therefore, organ protection treatment approaches have recently gained extensive popularity. Nowadays, combined cisplatin and irradiation treatment is a preferable strategy for head and neck cancer and organ preservation. However, this widespread approach has several adverse effects that reduce the efficacy of treatment, such as non-specific toxicity toward normal cells. Although this preferable treatment method has advantages, this non-surgical intervention may result in unwanted side effects, such as ototoxicity. It is reported that there are three main target mechanisms for prevention of ototoxic injury: 1) prevention of formation of reactive oxygen species; 2) neutralization of toxic products; and 3) inhibition of apoptosis pathways [1-2].

Cisplatin ototoxicity may originate from free radical-mediated damaged to various tissues, including the inner ear [3, 4]. Both irradiation and cisplatin have the potential to damage the cell by similar oxidative mechanisms [5]. Therefore, the use of antioxidants has become an alternative to prevent damage caused by tumor therapy because of research on natural antioxidants, which have been described as disease preventers.

Pomegranate (*Punica granatum*) is one of the oldest natural antioxidant fruits and is widely grown in many countries [6]. Kotamballi et al. [7] reported in their in vivo study that pomegranate extracts have antioxidant capacity.

Although many studies performed on ototoxicity originated from both radiation and cisplatin, the effects of fresh pomegranate juice (FPJ) on the ototoxicity after cisplatin, irradiation, and cisplatin-based chemo-irradiation have not been investigated suffi-

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Submitted: 20.02.2014 **Accepted:** 03.04.2014

ciently. Despite the use of pomegranate extract in many studies, the freshly squeezed form of it was not used in the ototoxicity studies [7, 8]. Therefore, we investigated the protective effect of short-term usage of fresh pomegranate juice (FPJ) on ototoxicity after irradiation, cisplatin, and cisplatin-based chemo-irradiation in this study because of insufficient information related to the preventive effects of short usage of fresh pomegranate juice and ototoxicity.

MATERIALS and METHODS

All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals issued by the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council [9].The study was also approved by the Dicle University Medical institutional ethics committee with the number of 2012/60.

Experimental Design

Fifty-six male adult Wistar Albino rats weighing between 220 and 320 grams were used in this study. All animal weights were measured before and at the end of the study. They were divided randomly into 8 groups, for a sample size of 7. The rats were assigned to sham control; FPJ; irradiation; cisplatin; irradiation plus FPJ; cisplatin plus FPJ; both cisplatin and irradiation; and combined use of cisplatin, irradiation, and FPJ. Group 1, which is the sham group, received saline placebo, whereas group 2 received only FPJ (2 cc/kg via gavage, 8 days). Group 3 was only exposed to ionizing radiation (7 Gy/radiation single fractionated [15] dosage). However, group 4 was treated with a single cisplatin dose at 8 mg/kg, while group 5 was treated with both irradiation (7 Gy/radiation single fractionated dosage) and FPJ (2 cc/kg via gavage, 8 days). Group 6 was treated with both single cisplatin (8 mg/kg) and FPJ (2 cc/kg via gavage, pre usage of cisplatin and radiation 5 days and 5 days after post-cisplatin), while group 7 was treated with a concomitant single cisplatin dose at 8 mg/kg and irradiation (7 Gy/radiation single fractionated dosage). In the last experimental group, group 8, combined use of cisplatin (8 mg/kg), irradiation (7 Gy/radiation single fractionated dosage), and FPJ (2 cc/kg via gavage) was applied to the rats.

Exclusion Criteria

Animals that showed signs of ear diseases were excluded. Other exclusion criteria were as follows: 1) otoscopically detectable external ear abnormalities, 2) signs of middle ear disease, and 3) absence of distortion product-evoked otoacoustic emissions at any of the frequency ranges tested.

Drugs and Anesthesia

Animals were anesthetized with 30 mg/kg of ketamine hydrochloride (Ketalar, Eczacibasi Ilac Sanayi ve Ticaret A.S., Lüleburgaz, Turkey) and 4 mg/kg of xylazine (Alfazyne 2%, Alfasan International B.V., Woerden, Netherlands). Cisplatin (Cisplatin DBL, Faulding Pharmaceuticals, Warwickshire, UK), at 8 mg/kg, was also administered intraperitoneally (ip). FPJ was given to animals at 2 cc/kg via gavage every day. The first dose of FPJ was given 5 days before cisplatin and irradiation administration and continued until sacrifice (5 days before and 5 days after cisplatin or radiation).

Total Phenolic Contents

Fresh pomegranate juice was assessed for phenolics and flavonoids. The rats were fed with FPJ, which was obtained just before the animal assays. One pomegranate fruit was squeezed with a juice extractor, and then the mixture was sieved to get FPJ just before determining the phenolic and flavonoid contents. The total content of polyphenols in the FPJ was determined according to the Folin-Ciocalteu method [10]. Quantification was plotted on a standard curve of gallic acid, and the results were expressed as gallic acid equivalents (GAE) (mg GAE/ 0.5 mL of FPJ). The total amount of phenolics was found to be 28.26 mg in 0.5 mL of FPJ.

Total Flavonoid Contents

The total flavonoid content of FPJ was determined by a colorimetric method based on the formation of flavonoid–aluminum complex [11]. The total flavonoid content was expressed as μ quercetin equivalents per 0.5 mL FPJ (μg QUE/0.5 mL FPJ). The total amount of flavonoid was found to be 36.51 μg in 0.5 mL of FPJ.

Sample Preparation for Tissue Assays

The cochlea samples were homogenized with 100 mM KH_2PO_4 buffer containing 1 mM EDTA (pH 7.4) and centrifuged at 4500 rpm for 30 min at 4°C. The supernatant was collected and kept at -20°C until use.

Protein Determination

Protein contents in the supernatant of cochlea samples were estimated by the method of Lowry et al. [12] using the Folin reagent and BSA as standard [10].

Determination of Carbonyl Contents

Carbonyl contents of oxidatively modified proteins were determined by the 2, 4-dinitro-phenylhydrazine (DNPH) method described by Levine et al. with some modifications [11]. The results were expressed as nanomoles of DNPH incorporated/mg protein based on a molar extinction coefficient of $22{,}000^{-1}cm^{-1}$.

Cochlear Dissection

After sacrifice of the animals, both cochlear and paracochlear tissues of each one were removed. By positioning the clamp in the external auditory canal, all bone parts of the leaflet were broken in a single movement, exposing the cochlea. All paracochlear tissues were removed from the cochleas to determine the protein carbonyl before histopathological process. Then, the cochleas were placed in 10% formaldehyde for standard histopathological processes.

Radiation Procedure

Prior to irradiation, the rats were anesthetized. Irradiation was delivered by an ALCYON-II model cobalt-60 teletherapy unit (General Electric (GE) Medical Systems, Codex, Paris, France) at a source-surface distance of 80 cm. A single dose of 7 Gy of radiation was given at a depth of 1 cm (thickness) with a dose rate of 0.4 Gy/min to an area of 30 x 30 cm of the head of the rat in supine position. The radiation was limited to the head, centered across the cochlea to spare the rest of the body.

Hearing Assessment

At the beginning of the study, which was before drug administration, rats were deeply anesthetized with ketamine (50 mg/kg-) plus xylazine (10 mg/kg) intraperitoneally to perform the first DPOAE measurement. At the end of the study, rats were again anesthetized for a second DPOAE test. Hearing status of all animals was also deter-

mined by DP gram with the signal-to-noise ratio (SNR). DPOA was measured in both ears of each rat.

To test the integrity of the hair cells, all animals were tested with DPOAEs in an isolated room (less than 50 dB background noise). Otomicroscopy was performed to confirm that the external auditory canal and tympanic membrane were normal before each measurement. The otoacoustic emissions were performed by using a standard commercial ILO-96 OAE apparatus cochlear emission analyzer (Otodynamics Ltd, London, UK). The data were processed and evaluated with software (EZ Screen 2 Otodynamics OAE Screening and Data Management Software, Hatfield, UK). Each DPOAE test required about 3 minutes. The primary tones were introduced through an inserted earphone using a plastic adapter that sealed the probe in the outer ear canal of the animal. The stimulus consisted of two pure tones, f1 (65 dB) and f2 (55 dB), fixed at f1/f2 = 1.22 at 70 dB SPL. DPOA values were evaluated at 2, 4, 6, and 8 kHz and considered positive for signal-to-noise ratios of 6 dB, as specified by the manufacturer. Hearing status of all animals was also determined by DP gram with the signal-to-noise ratio (SNR). The frequencies of SNR were measured at 1.0 kHz 1.4 kHz, 2.0 kHz, 2.8 kHz, and 4.0 kHz.

Histopathologic Examination

The cochleas of each rat were fixed for 24 hours in 10% formaldehyde solution and subjected to decalcification for 3 weeks in 5% formic acid solution. After the fixation and decalcification processes, the cochleas were washed with tap water for 24 hours, dehydrated with a graded alcohol series, and subsequently paraffinized. Each paraffin-embedded specimen was sectioned with a microtome (Leica RM-2125RTS) at a thickness of 4 µm and stained with H&E, followed by analysis with a Nikon ECLIPSE 80i microscope.

In the morphological analysis, less than 10% was accepted as mild (score: 1), 10%-50% was defined as moderate (score: 2), and damaged hair cell area of the section of more than 50% was defined as severe (score: 3).

Statistical Analysis

Statistical evaluation was carried out using Statistical Package for the Social Sciences (SPSS) version 15.0 for Windows Inc. (Chicago, IL, USA) Results were analyzed statistically by Kruskal-Wallis test to determine differences in amplitudes and SNRs of DPOAEs and corresponding noise floor differences and thresholds for each frequency. The histopathological variations and protein carbonyl values of the eight groups were evaluated by Mann-Whitney U-test. Statistical significance was accepted as a p value of less than 0.05.

RESULTS

Histopathological Results

According to our results, H&E staining showed no observed distinctive difference in cochlear hair cells between the sham control and the FPJ group (p=0.591) (Figure 1a, b). In the cisplatin-treated rats, hair cells showed serious hair loss and degeneration of hair cells (p=0.004) (Figure 2a). The results of the cisplatin plus FPJ group showed that FPJ protected hair cells (p=0.010) (Figure 2b). Severe degeneration of hair cells was also observed in the cisplatin plus irradiation group (p=0.058) (Figure 3a). Although some deterioration was

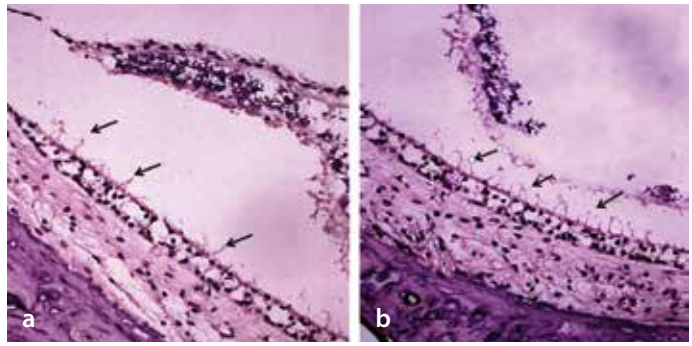

Figure 1. a, b. Three light micrographs of hair cell cross-sectional area through the basal turn. The sham group showing normal appearance of three external ciliated cells (*arrows*) (a); FPJ-treated group showing the normal appearance of hair cells (*arrows*) (b)

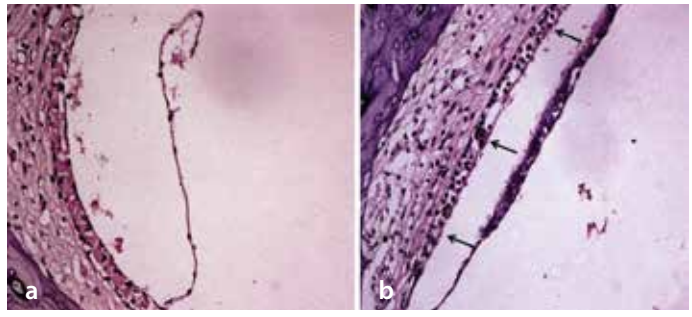

Figure 2. a, b. Cisplatin-treated group showing the degeneration of hair cells (*arrow*) (hematoxylin & eosin, X400) (a); cisplatin plus FPJ group showing the normal appearance of hair cells (hematoxylin & eosin, X400) (b)

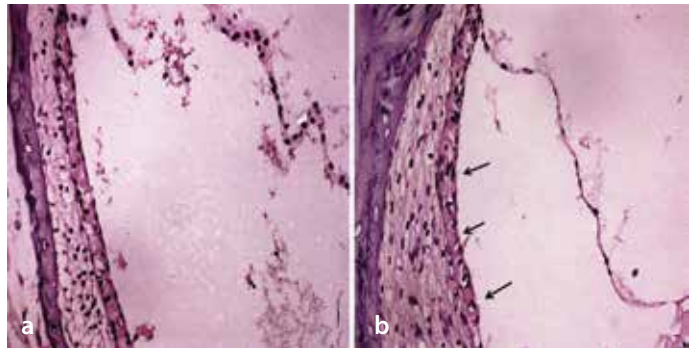

Figure 3. a, b. Irradiation-treated group showing the degeneration of hair cells (*arrow*) (hematoxylin & eosin, X400) (a). Irradiation plus FPJ group showing normal appearance and degeneration of hair cells (hematoxylin & eosin, X400) (b)

observed in the irradiation plus FPJ group, hair cells were moderately preserved by FPJ (Figure 3b). Hair cells demonstrated severe degeneration in the irradiation group (p=0.0026) (Figure 4a). The results of combined use of irradiation plus cisplatin plus FPJ also indicated that FPJ mostly protected the hair cells (p=0.006) (Figure 4b). Vacuolization and nuclear degeneration in spiral ganglion cells (SGCs) were observed in the cisplatin, irradiation, and cisplatin plus irradiation groups (Figure 5). The results related to the protective effect of FPJ are given in Figure 6.

Hearing Results

The analysis of the SNR emission results revealed statistically significant differences between the irradiation and sham control groups (p=0.034 and p=0.028 at frequency 1.0 and 1.4 mHz); irradiation and

irradiation plus FPJ groups (p=0.025 at frequency 1.0 mHz); sham control and cisplatin plus irradiation groups (p=0.048 at frequency 1.0; p=0.018 at frequency 2.0 mHz; p=0.018 at frequency 2.8 mHz; p=0.028 at frequency 4.0); cisplatin plus irradiation and combined use of cisplatin, irradiation, and FPJ groups (0.018 at frequency 1.0 mHz and 0.026 at frequency 1.4 mHz). The results on SNR values have been given in Figure 7.

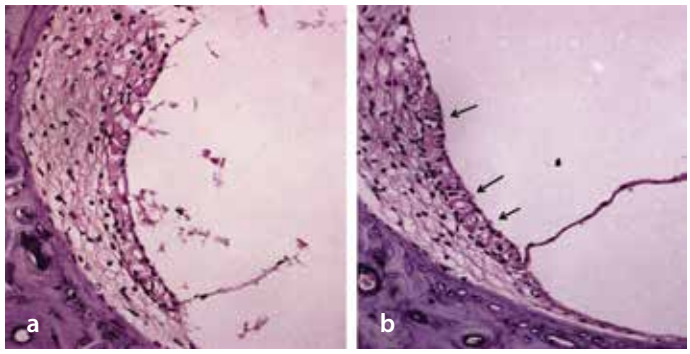

Figure 4. a, b. Cisplatin plus irradiation-treated group showing the severe degeneration of hair cells (*arrow*) (hematoxylin & eosin, X400) (a). Combined use of cisplatin plus irradiation and FPJ group showing protected hair cells (*arrow*) (hematoxylin & eosin, X400) (b)

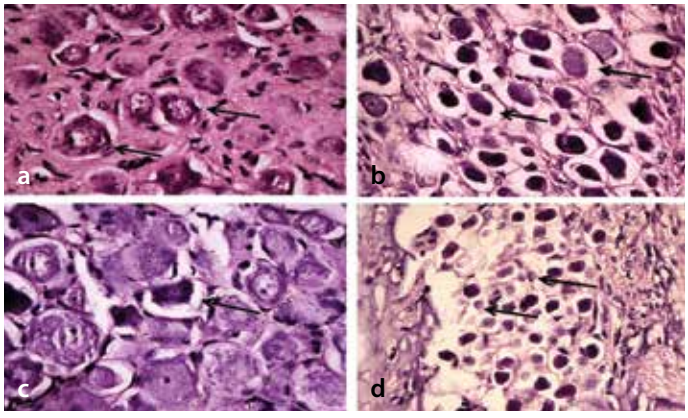

Figure 5. a-d. Sham group showing the normal appearance of spiral ganglion cells (*arrows*) (hematoxylin & eosin, X400) (a). Cisplatin-treated group showing eosinophilic cytoplasm of spiral ganglion cells; spiral ganglion cells nuclei are not seen even in areas that can be viewed with severe degeneration (b). Cisplatin plus FPJ group showing some vacuolization and nuclear degeneration seen in some areas (*arrow*) (hematoxylin & eosin, X400) (c). İrradiation plus FPJ group 5 showing vacuolization and nuclear degeneration in some areas and protection in some spiral ganglion cells (*arrow*) (hematoxylin & eosin, X400) (d)

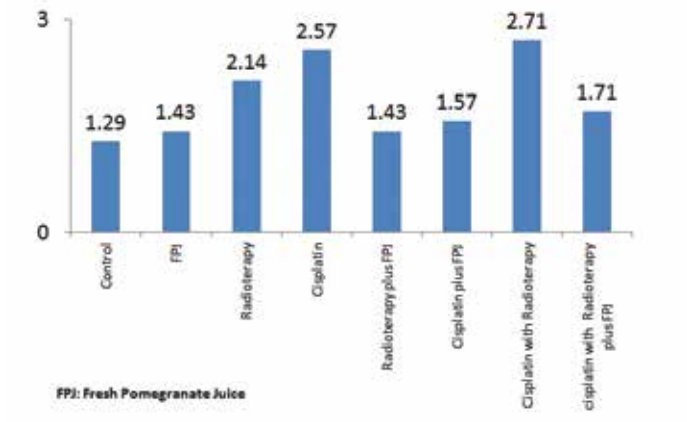

Figure 6. Histopathological comparisons between the groups

Protein Carbonyl Content

The result of the comparison between the sham control and experimental groups indicated that irradiation increased protein carbonyl content in paracochlear tissues dramatically (p<.001). However, another interesting result of the study is the decrease of protein carbonyl in the rats treated by both cisplatin and irradiation. The third result is the decrease of the protein carbonyl content in both irradiation- and fresh pomegranate juice-treated groups. It is obvious that FPJ dramatically decreased the protein carbonyl content that originated from irradiation (p<.001). Furthermore, any supportive effect of FPJ was not observed when the cisplatin and cisplatin plus FPJ groups were compared. For more information, please see Table 1 and Figure 8.

DISCUSSION

Cisplatin is a platinum-based chemotherapy drug widely used to treat a variety of malignant tumors, including neck tumors. However,

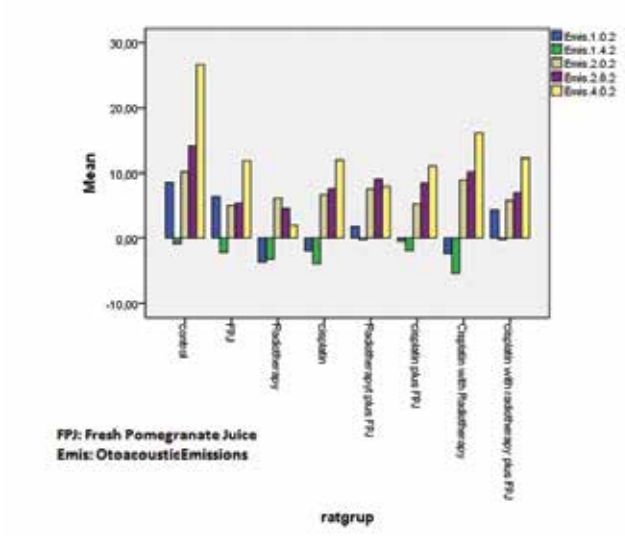

Figure 7. Comparison of otoacoustic emissions, which are shown as SNR alterations between groups

Table 1. The result related to the protein carbonyl content in the paracochlear tissue

Groups	n	Mean ± SD	Compared Groups	p
Sham	7	793.63±0.94	Sham - FPJ	<.001
FPJ	7	429.31±0.00	Sham - RT	<.001
Radiotherapy (RT)	7	3264.11±1.52	Sham - Cisplatin	<.05
Cisplatin (Cis)	7	461.99±114.02	Sham - RT+FPJ	<.001
RT+FPJ	7	350.07±15.38	Sham - Cis+FPJ	<.001
Cis+FPJ	7	455.59±1.41	Sham - Cis+RT	<.001
Cis+RT	7	84.94±12.09	Sham - Cis+RT+FPJ	<.001
Cis+RT+FPJ	7	493.16±.78	RT - RT+FPJ	<.001
			Cis-Cis+FPJ	>.05
			Cis+RT - Cis+RT+FPJ	<.001

FPJ: fresh pomegranate juice; RT: irradiation; Cis: cisplatin; SD: standard deviation

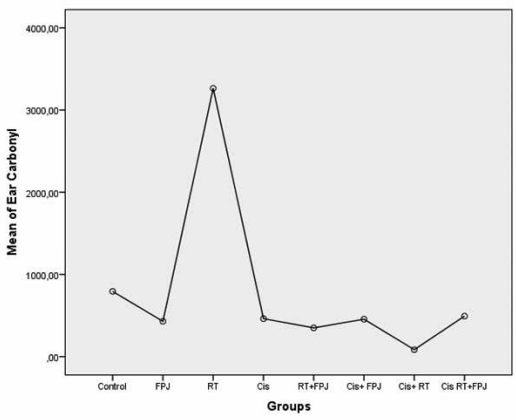

Figure 8. The result related to the protein carbonyl content in the paracochlear tissue according to different groups

cisplatin is highly toxic to the inner ear. Cisplatin-induced ototoxicity is initiated by its uptake into the sensory hair cells, neurons, and/or supporting cells in the inner ear [12]. Therefore, reducing the side effects of cisplatin, such as ototoxicity, is a very important point. Teker AM et al. [8] stated that the ototoxic side effects of cisplatin can not be decreased by traditional medical treatments. However, they indicated that the antioxidant capacity of pomegranate extract has protective effects against cisplatin-induced ototoxicity. Additionally, they suggested that DPOAE is a hearing assessment method that represents the function of the outer hair cell, one of the targets damaged by cisplatin.

It is really reported that all juices have potential to protect cells from oxidative damage but have differences that could originate from the different phenolic contents and patterns. One of the juices stated here is pomegranate. Pomegranate is widely consumed in fresh and beverage forms and has gained widespread popularity as a healthy valued food. Pomegranate juice, obtained by pressing the whole fruit or arils, is a rich source of phenolic compounds. Pomegranate has been shown to have potent in vitro antioxidant capacity, in vivo anti-atherosclerotic properties, anti-viral protections, anti-hypertensive activities, and anti-cancer effects [13]. Moreover, the phenolic antioxidants present in pomegranate have been shown to contribute to the decrease of oxidative stress. New information on the positive effects of pomegranate and the public awareness of the impact of food on health have greatly expanded the demand for the pomegranate fruit and its by-products in the Western world [13]. Pomegranate has been illustrated to possess anti-carcinogenic properties that may suppress various cancers. Many research applications have been made to determine the chemopreventive effects of pomegranate on some types of cancer, such as lung, prostate, and bladder cancer [14]. It has been indicated that methanolic extract of pomegranate peel could inhibit aluminum-induced oxidative stress and histopathological alternations in brains of female rats, and Di Nunzio et al [15] also stated that these effects may be related to anti-apoptotic and antioxidant activities. Similarly, Kumar et al. [16] indicated that pomegranate (*Punica granatum*) peel extract provides protection against mercuric chloride-induced oxidative stress in Wistar strain rats. It is also reported that pomegranate extracts have been found to have strong anti-inflammatory, antioxidant, and even antitumor properties in vivo and in vitro [17].

In our study, we used fresh pomegranate juice (FPJ) instead of pomegranate extract (PE). Therefore, the form of pomegranate used in our study is different than in Teker AM et al. [8], because they used Pomegranate Extract in their study. However, we similarly observed that FPJ has protective effects on hair cells in Inner and ototoxicity. On the other hand, the methodology of our study is more comprehensive, because we investigated the protective effects of FPJ on ototoxicity originating from cisplatin, irradiation, and combined use of cisplatin and irradiation. As it is known, radiotherapy is widely used for the treatment of neck cancers, and cochlear hair cell death is regarded to be responsible for radiation-induced sensorineural hearing loss (SNHL), which is one of the principal complications of radiotherapy (RT) for head and neck cancers [18].

In our study, we used different analysis methods, such as DPOAE, histopathology, and protein carbonyl, which is a sensible indicator of protein oxidation-that is, an oxidative stress indicator to know whether FPJ has protective effects on hair cells in the inner ear or ototoxicity.

As it is stated in the studies discussed above, FPJ contributes to decreased oxidative stress and behaves as an anticarcinogenic agent. However, no study performed with fresh pomegranate juice is available. Therefore, an originality of this study is to investigate the protective effects of fresh pomegranate juice. We observed in our laboratory that the phenolic and flavonoid contents of FPJ are much higher than waited of the pomegranate juice for ten minutes. For instance, the phenolic content of FPJ was 56.72 µg GAE/µL while the waited of pomegranate juice was 38.87 µg GAE/µL. Similarly, the flavonoid content was 51 µg GAE/µL in FPJ while 40.68 µg GAE/µL in waited ten minutes of pomegranate juice. Therefore, it can be stated that FPJ is more effective than other forms.

Gayathri et al. [19] reported that protein carbonyl (PC) is considered an excellent biomarker of oxidative protein damage, as proteins are susceptible to oxidative modification. Also, Almroth et al. [20] reported that the connection of the carbonyl group in proteins is clearly proportional to the protein subjected to oxidative damage, and an increase in carbonyl content is associated with various pathological disorders. We observed in our study that irradiation dramatically increased PC content, whereas FPJ blocked and decreased PC content strikingly. Another interesting result on the anti-oxidative capacity of FPJ is the decrease in PC in the FPJ-treated group versus sham group. In general, we can state that FPJ plays an important role to decrease PC originating from oxidative stress. However, according to the comparison of cisplatin plus irradiation and the combined use of cisplatin, we can state that FPJ behaves as a regulator (Table 1 and Figure 8).

There are many studies of cisplatin-induced ototoxicity via DPOAE. Ototoxicity may occur within hours to days after treatment with cisplatin. Hearing loss appears to be dose-related, cumulative, bilateral, and usually permanent and occurs initially at higher frequencies [21]. In our study, single-dosage (8 mg/kg) cisplatin deteriorated the cochlea histopathologically. The results showed that cochlear hair cells were fully deteriorated in the cisplatin plus irradiation group. But, FPJ obviously protected cochlear hair cells against cisplatin and cisplatin plus irradiation treatment. The SNR values and protein carbonyl content measured in this study supported our histopathological re-

sults. Our histopathological results are supported by Teker AM et al. [8] (Figure 1-6).

Low et al. [22] reported that irradiation for head and neck cancers can potentially cause hearing loss. Because the cochlea and auditory pathways are often included in the radiation fields. The results of this study indicated that hearing loss was only observed at DPOAE frequencies, such as 1.0 and 1.4. However, the results of FPJ treatment showed that FPJ prevented hearing loss originating from irradiation and cisplatin. Akmansu et al. [23] reported that sub-lethal dose (5.5 Gy) irradiation causes hearing loss. Our DPOAE results were consistent with the results of Akmansu et al.

In conclusion, the results of this study indicate that fresh pomegranate juice has important capacity to eliminate damage to cochlear hair cells resulting from irradiation, cisplatin, and combined use of cisplatin and irradiation. However, protein carbonyl can be used as a biomarker for ototoxicity. Finally, more performance is necessary to prove the effectiveness of fresh pomegranate juice, which contains more phenolics and flavonoids.

Ethics Committe Approval: Ethics committee approval was received for this study from the ethics committee of Dicle University Medical institution (Date: 2012, Document no: 60).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - M.A., S.D.; Design - M.A., A.G.; Supervision - M.A., S.D.; Funding - M.A., U.A.; Materials - M.A., S.B.Z., S.A., U.A., I.K.; Data Collection and/or Processing - M.A., B.Y., I.K.

Acknowledgments: The authors thank to Professor Ömer Satıcı, from the Department of Bioistatistics of Dicle University Faculty of Medicine, for his valuable statistical assistance and to Professor Murat Kızıl from Department of chemistry, Dicle University Faculty of Medicine for his valuable biochemical analyses and DUBAP Editing for editing of English .

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This study was supported by Dicle University Scientific Found (DUBAP, project number13-TF-94)

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