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Original Article

Evaluation of Lapatinib and Trastuzumab for Ototoxic Effects

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OBJECTIVE: Trastuzumab and lapatinib are widely used chemotherapeutic agents. Our aim in this study was to assess the possible ototoxicity of these chemotherapeutic agents.

MATERIALS and METHODS: Forty-eight rats were divided into six groups: Group 1 (control, n=8) received intraperitoneal saline for 7 days. Group 2 (n=8) and Group 3 (n=8) received 10 mg/kg and 30 mg/kg single doses of intraperitoneal trastuzumab, respectively. Lapatinib was administered by oral gavage to Group 4 (n=8) at 100 mg/kg/day and to group 5 (n=8) at 300 mg/kg/day for 7 days. Group 6 (n=8) received only one dose of 10 mg/kg intraperitoneal trastuzumab; subsequently, Group 6 received one dose of lapatinib at 100 mg/kg/day by oral gavage for 7 days. Before any medication was administered, distortion product emissions (DPOAE) were obtained. DPOAE tests were performed again on the rats on day 7, after which the mastoid bullas were harvested. The apoptosis degree was evaluated by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) procedure.

RESULTS: The lapatinib 300 and lapatinib+trastuzumab groups (p=0.008 and p=0.001, respectively) were significantly different from the control group according to the spiral ganglion TUNEL. Apoptosis in the organ of corti was statistically different compared with the control group in the lapatinib 100, lapatinib 300, and lapatinib+trastuzumab groups (p=0.035, p=0.001, and p<0.001, respectively). Trastuzumab induced damage in only the organ of corti; however, lapatinib induced damage in both the organ of corti and spiral ganglion. The degree of the damage in the organ of corti was high when trastuzumab and lapatinib were concomitantly used. Supporting this data, a reduction in DPOAE amplitudes was observed during the combined usage of the drugs.

CONCLUSION: Administering trastuzumab and lapatinib causes ototoxic effects.

KEYWORDS: Lapatinib, ototoxicity, trastuzumab

INTRODUCTION

Trastuzumab (Herceptin®; Genentech, California, San Francisco, USA) is used for treating human epidermal growth factor receptor 2 (HER2)-positive breast cancers. Trastuzumab is a humanized recombinant monoclonal antibody that acts on the extracellular portion of the HER2 protein [1]. Lapatinib (Tykerb®; Glaxo-Smith Kline) is a tyrosine kinase inhibitor that suppresses epidermal growth factor 1 (EGF-1) and intracellular phosphorylation of the HER-2/neu receptor. In studies with trastuzumab, cardiotoxicity is stated as the most common side effect [2]. The known side effects of lapatinib are the reduction of the left ventricular ejection fraction, interstitial lung disease/pneumonitis, dizziness, fatigue, severe diarrhea, dry cough, white sores in the mouth or lips, nosebleeds, nausea, stomach pain, jaundice, loss of appetite, aspartate aminotransferase (AST) changes, alanine aminotransferase (ALT) changes, and hematological changes [3, 4]. To our knowledge, the ototoxicity of these chemotherapeutic agents has not yet been studied. The aim of this study is to identify whether these agents are ototoxic in rats and to determine the dose dependency of the ototoxicity.

MATERIALS and METHODS

The study was approved by the Animal Experiments Ethical Committee of Adnan Menderes University (64583101/2013/038). A total of 48 male rats aged 4–8 months were randomly divided into six groups. All rats were subjected to distortion product otoacoustic emissions (DPOAE) on the first day after being anesthetized with ketamine/xylazine. Group 1 (control group) received

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intraperitoneal injections of saline for a period of 7 days. Group 2 and Group 3 received 10 mg/kg and 30 mg/kg single doses of intraperitoneal injections of trastuzumab, respectively. Lapatinib was administered to Group 4 with a dose of 100 mg/kg/day by oral gavage for 7 days. Group 5 received lapatinib with a dose of 300 mg/kg/day by oral gavage for 7 days. Group 6 received a single dose of 10 mg/kg intraperitoneal trastuzumab; lapatinib was administered with a dose of 100 mg/kg/day once by oral gavage for 7 days. On the 7th day, the DPOAE test was repeated in rats under ketamine/xylazine anesthesia, and the rats were then sacrificed by cervical dislocation. The mastoid bullas were immediately harvested right after cervical dislocation and were maintained in 10% formalin for histopathologic examination.

Auditory Assessment

First, the tympanic membranes and external auditory canals of the rats were examined. Rats with problems in the external or middle ear were excluded. All DPOAE tests were performed in a quiet room after ketamine/xylazine anesthesia on the first day (before giving any medication) and were repeated on the 7th day. The DPOAE test was applied to the left ear of each rat with Otodynamics Echoport cochlear emissions, and the results were analyzed using Otodynamics ILO software (MAICO MI 34; Berlin, Germany). Frequencies of 1, 1.5, 2, 3, 4, 6, and 8 kHz were studied. The stimulus was two pure tones (F1, F2; F1/F2=1.22) at a sound pressure level of 70 dB. The signal-to-noise ratio was recorded.

Histopathological Procedures

Samples of inner ear tissues were prepared for examination with light microscopy. After fixing the samples by immersion in 10% phosphate buffered formalin (pH 7.4) overnight, they were dehydrated in concentrations of ethanol (60%, 70%, 80%, and 90% of absolute ethanol) and xylene. The samples were then embedded in paraffin blocks. Serial sections (5 µm thick) were cut from the paraffin blocks using a Leica RM2255 rotary microtome (Germany). Deparaffinizing, hydrating, and staining with hematoxylin-eosin were then applied. Damage of the inner tissue samples was revealed by light microscopic evaluation. Also, all of the samples were stained with the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) method.

TUNEL Procedure

The degree of apoptosis was assessed using the TUNEL procedure with an apoptosis detection kit. The procedure was performed with an *in situ* Cell Death Detection Kit, POD (Cat No. S7102; Merck Millipore, Germany) following the company's protocol. Serial 5-µm thick paraffin-embedded sections were deparaffinized, rehydrated in graded alcohol, and washed in distilled water, followed by PBS. Deparaffinized tissue sections were incubated with 20 µg/mL proteinase K for 30 min at 37°C. Then the sections were rinsed and incubated with equilibration buffer (1×) for 30 min at room temperature. Digoxigenin-labeled deoxynucleotide (dNTP) tail was incubated with terminal deoxynucleotidyl (Tdt) transferase for 60 min at 37°C and washed with buffer for 10 min at room temperature. Next, the tissue sections were incubated with anti-digoxigenin peroxidase antibody at room temperature for 30 min. After that, the tissue sections were stained with diaminobenzidine (DAB). Staining was assessed with a light microscope following counterstaining with hemotoxylin. For each slide, five fields were chosen at random and the number of TUNEL-positive cells was determined per field. The apoptotic index (apoptotic nuclei percentage) was calculated as: Apoptotic index=apoptotic nuclei/total nuclei count×100. All counting evaluations were done blindly.

Statistical Analysis

The data of DPOAE amplitude and spiral ganglion cochlea apoptosis were expressed as mean percentiles. The data were analyzed with Statistical Package for the Social Sciences (SPSS) 19.0 statistical software (SPSS Inc.; 10241440, İstanbul, Turkey). Friedman's two way analysis of variance by ranks was used to compare the data from the related samples. For the independent samples, the Kruskal–Wallis test and pairwise comparisons of groups were used. The significance level of $p<0.05$ was used in all statistical analyses.

RESULTS

DPOAE Values

No statistically significant differences between DPOAE values from the pre- and post-treatment tests were found in the control group ($p>0.05$). The initial DPOAE results before treatment presented comparable values in all groups ($p>0.05$).

Statistically significant differences in DPOAE amplitudes from the post-treatment test among all groups were found at 1.5 Khz ($p=0.046$), at 3 kHz ($p=0.013$), at 6 kHz ($p=0.016$) and 8 kHz ($p=0.002$). There was no statistically significant difference in the post-treatment DPOAE amplitudes at 1 kHz, 2 kHz, and 4 kHz ($p>0.05$). A comparison of the mean amplitudes of the post-treatment DPOAE tests are shown in Figure 1. Statistically significant differences in the DPOAE amplitudes acquired post-treatment were found at 1.5 kHz between the lapatinib 100 and control groups ($p=0.036$), the lapatinib+trastuzumab and control groups ($p=0.012$), and the trastuzumab 30 and control groups ($p=0.013$). The same differences at 6 kHz are detected between the trastuzumab 10 and trastuzumab 30 groups ($p=0.020$), the trastuzumab 10 and control groups ($p=0.006$), the lapatinib+trastuzumab and trastuzumab 30 groups ($p=0.045$), the lapatinib+trastuzumab and lapatinib 100 groups ($p=0.029$), and the lapatinib+trastuzumab and control groups ($p=0.015$). The highest DPOAE frequency was 8 kHz. Also, the differences at 8 kHz were the same between the trastuzumab 10 and trastuzumab 30 groups ($p=0.016$), the trastuzumab 10 and control groups ($p=0.002$), the lapatinib+trastuzumab and control groups ($p=0.004$), and the lapatinib 300 and control groups ($p=0.009$). Notably, the lapatinib+trastuzumab group showed marked ototoxicity compared with the control group at 1.5, 3, 6, and 8 kHz.

The Histopathological Findings

Evaluation of the hematoxylin/eosin-stained sections showed normal morphologies of the spiral ganglion and organ of corti in the control group. The low and high dose trastuzumab groups (Group 2 and 3) had normal morphological appearance of the spiral ganglion. However, some sections possibly showed mild degenerative changes in the organ of corti. The low and high dose lapatinib groups (Group 4 and 5) also possibly showed mild degenerative changes in the organ of corti. The low dose lapatinib group (Group 4) had no degeneration in the spiral ganglion; however, the high dose lapatinib group (Group 5) had degeneration in the spiral ganglion. The lapatinib+trastuzum-

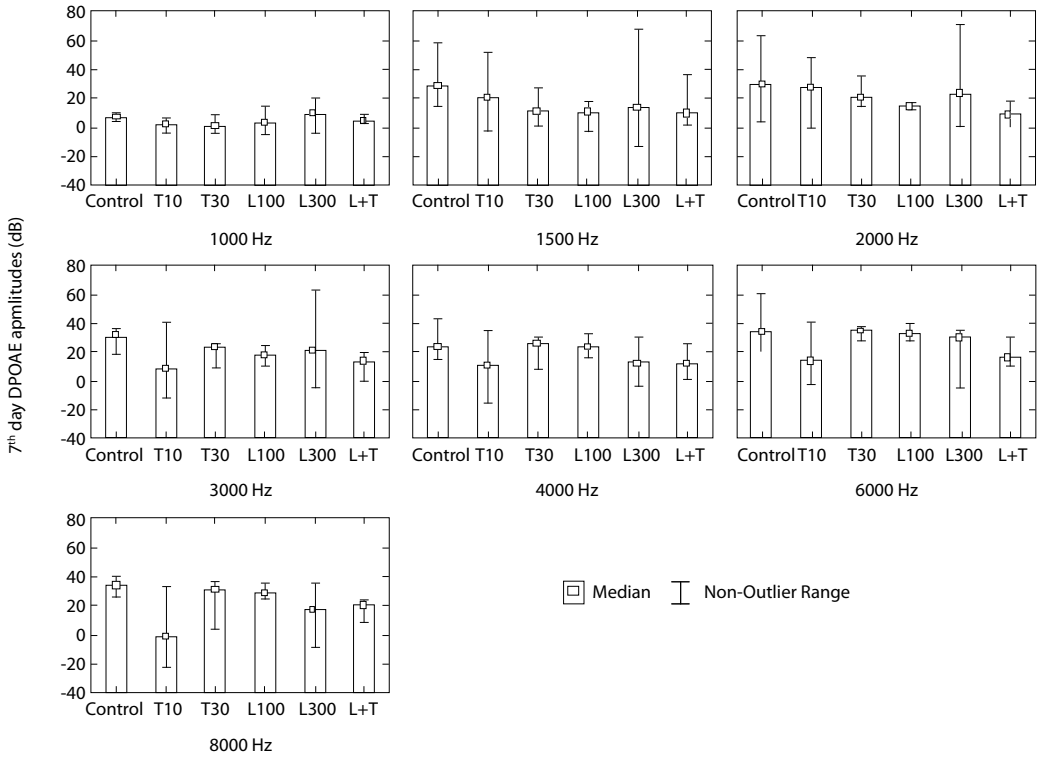

Figure 1 a-g. Comparison of DPOAE mean amplitudes at the seventh day of all groups: at 1000 Hz (a), at 1500 Hz (b), at 2000 Hz (c), at 3000 Hz (d), at 4000 Hz (e), at 6000 Hz (f), at 8000 Hz (g)

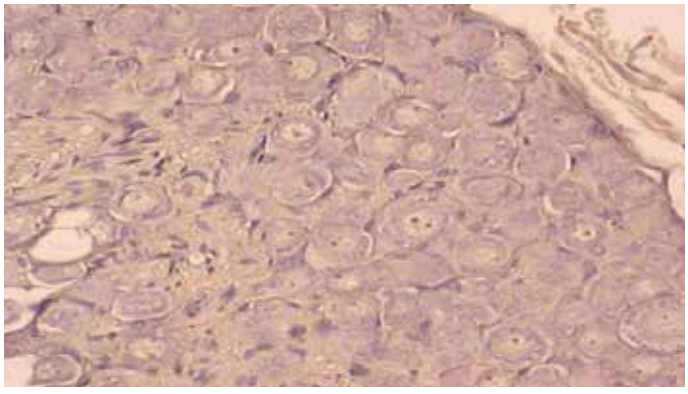

Figure 2. Transtuzumab (30 mg; Group 2) spiral ganglion TUNEL staining: no apoptosis or tissue damage is observed (200×)

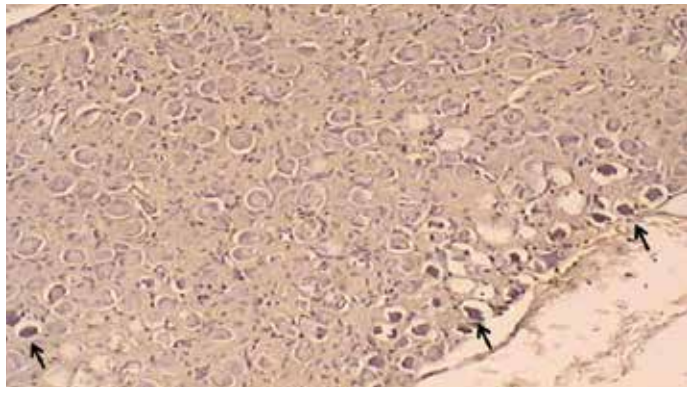

Figure 3. Lapatinib (100 mg; Group 4) spiral ganglion TUNEL staining: low apoptosis and tissue damage are observed (arrow) (100×)

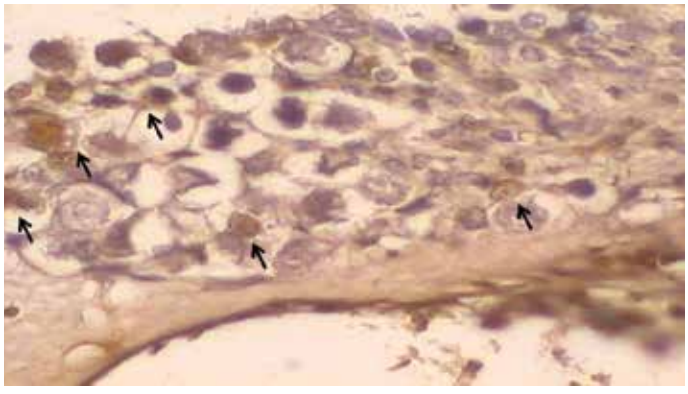

Figure 4. Lapatinib (300 mg; Group 5) spiral ganglion TUNEL staining: prominent apoptosis and tissue damage are observed (arrow) (400×)

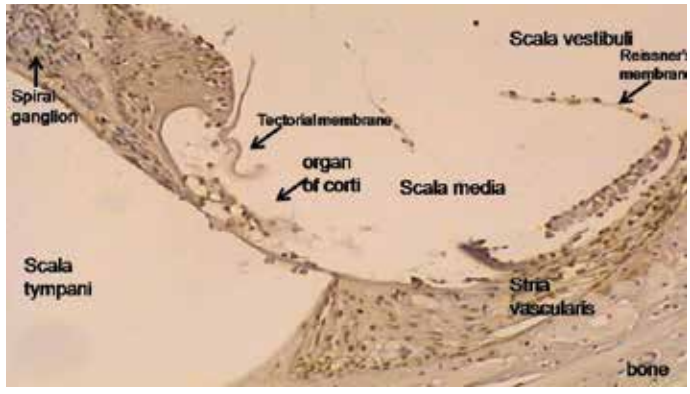

Figure 5. Lapatinib+trastuzumab (Group 6) cochlea TUNEL staining: high apoptosis is observed (100×)

Table 1. Comparison of the spiral ganglion and organ of corti tunnel apoptosis median scores (25–27 percentiles) of the groups

	Control	Trastuzumab 10	Trastuzumab 30	Lapatinib 100	Lapatinib 300	Lapatinib+trastuzmab	p value
Spiral ganglion	2.00 (1.00–2.00)	2.00 (1.00–2.50)	2.00 (2.00–2.75)	2.00 (1.00–2.00)	19.00 (17.25–20.75)	20.50 (19.00–22.00)	<0.001
Organ of corti	1.50 (1.00–2.00)	18.00 (14.50–20.50)	17.50 (14.25–21.75)	19.50 (18.00–22.50)	22.00 (20.25–24.75)	41.50 (37.50–44.50)	<0.001

*Kruskal–Wallis test

ab group (Group 6) had degeneration both in the organ of corti and the spiral ganglion.

The apoptotic indices corresponding to the experimental groups were measured by TUNEL staining in the spiral ganglion and the organ of corti (Figures 2-5). The values were compared with the Kruskal–Wallis test (Table 1).

There was no apoptosis detected in the spiral ganglion and organ of corti in the control group.

The lapatinib 300 and lapatinib+trastuzumab groups (p=0.008, p=0.001) were significantly different from the control group with regard to the spiral ganglion TUNEL staining (Figures 4, 5). The lapatinib+trastuzumab group was significantly different from the trastuzumab 10 and 30 groups with regard to the spiral ganglion TUNEL assay (p=0.001, p=0.020). Damage in the spiral ganglion was observed in the lapatinib+trastuzumab group, but not in the groups with trastuzumab alone. Additionally, in the groups that received different doses of trastuzumab, there was no apoptosis in the spiral ganglion (Figure 2). Lapatinib caused more apoptosis in the spiral ganglion at higher doses (Figure 3).

According to the TUNEL assay values, the apoptosis in the organ of corti in the lapatinib 100, lapatinib 300, and lapatinib+trastuzumab groups were statistically different compared with the control group (p=0.035, p=0.001, p<0.001). The group with either 10 mg or 30 mg trastuzumab alone was not statistically different from the control group (p>0.05). The apoptosis of the organ of corti in the lapatinib+trastuzumab group when compared with the trastuzumab 10 or 30 groups, was statistically different (p=0.004 and p=0.013, respectively) (Figure 5). A statistical difference in apoptosis was not detected between the lapatinib 100 and 300 groups in the organ of corti. In addition, a statistical difference in apoptosis was not detected between the trastuzumab 10 and 30 groups (p>0.05). The trastuzumab dose did not change the magnitude of apoptosis.

Interestingly, there was no apoptosis detected in the semicircular canal and crista.

DISCUSSION

Ototoxicity is a side effect that can develop due to the use of chemotherapy drugs [5, 6]. Cisplatin is one of the drugs which is best known to have this side effect [7, 8]. To the best of our knowledge, trastuzumab and lapatinib have not been assessed for ototoxicity. Because these two chemotherapy drugs are used sequentially or combined with other chemotherapy drugs, clinical studies have not been planned. In the literature, erlotinib and imatinib dependent ototoxicity have been reported, as they are tyrosine kinase inhibitors that effect ototoxicity along a different pathway [1, 6, 9]. When we examine the studies relating tyrosine kinase inhibitors to hearing loss, many of them displayed mechanisms related to EGFR and HER [10, 11]. EGF-1 and HER2,3,4 receptors were found in the rat inner ear [10]. Also, EGFR has been found to be important in cell growth of the cochlea in rats [11]. Bilateral sensorineural hearing loss due to imatinib has been reported [9]. After daily oral usage of 400 mg imatinib, hearing loss developed on the 8th day. Examination revealed no additional pathology of the ear. Yet another case of sensorineural hearing loss due to the usage of imatinib was chronic myelogenous leukemia [6]. After a 400 mg/day dose of imatinib was administered for five days, bilateral hearing loss developed [6]. In addition to this, sensorineural hearing loss due to erlotinib was reported in a patient [12].

Because trastuzumab and lapatinib are rarely used alone, it is difficult to estimate which drug induces ototoxicity. In our study, we investigated whether the ototoxic effect increased with different doses or with the combined use of these drugs. Since clinical studies have been performed on the combined use of trastuzumab and lapatinib, we created similar experimental groups [13]. There were no previous reports on trastuzumab and lapatinib-associated ototoxicity. Therefore, the effect of the drug dose was determined, similarly to other toxicity studies [14, 15]. Intraperitoneal administration of only one dose of trastuzumab (10 mg/kg) was reported to induce cardiotoxicity in rats [14]. When determining the dose of lapatinib, a study was considered which investigated the involvement of the liver using a 100 mg/kg daily dose of latinib for one week via oral gavage followed by examination with gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-improved magnetic resonance imaging (MRI) [15]. In our study, "high dose" refers to the dose that is three times higher than the regular dose. One of the important findings of our study is that trastuzumab only damaged the organ of corti, while lapatinib damaged both the organ of corti and spiral ganglion. The damage to the organ of corti was particularly significant when trastuzumab and lapatinib were used concomitantly, suggesting a synergistic mechanism of the effects of trastuzumab on lapatinib. Supporting this data, a reduction in DPOAE amplitude was observed during combined usage.

These findings, notably the histopathological results in rats, support that trastuzumab and lapatinib treatment has ototoxic effects. Trastuzumab and lapatinib are commonly used during chemotherapy in clinical practice. According to our study, we recommend taking the hearing of chemotherapy patients into consideration. A thorough clinical study on the ototoxicity of these drugs when used as monotherapies may reveal further evidence that supports our work.

Ethics Committee Approval: Ethics committee approval was received for this study from Animal Experiments Ethical Committee of Adnan Menderes University (64583101/2013/038).

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Data Collection and/or Processing - A.E., B.D., N.E., S.A., B.E.; Analysis and/or Interpretation - A.E., İ.K.Ö, Y.B.; Literature Search - A.E., Y.B.; Writing Manuscript - A.E., B.D., C.G., Y.B.; Critical Review - A.E., C.G., Y.B., S.B.

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