Scanning Microscopy

Volume 9 | Number 1 Article 20

3-27-1995

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Scanning Microscopy, Vol. 9, No. 1, 1995 (Pages 271-281) 0891-7035/95$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

SEMIQUANTITATIVE ANALYSIS BY SCANNING ELECTRON MICROSCOPY OF COCHLEAR HAIR CELL DAMAGE BY OTOTOXIC DRUGS

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(Received for publication December 30, 1994 and in revised form March 27, 1995)

Abstract

The ototoxicity of cisplatin and carboplatin in the organ of Corti of the guinea pig was evaluated semiquantitatively. Damage of the stereocilia of outer hair cells (OHCs) observed by scanning electron microscopy (SEM) was classified into normal, grade 1 (10-50% loss of stereocilia), grade 2 (less than 50% remaining stereocilia), or grade 3 (missing stereocilia). The OHCs observed by light microscopy (LM) were classified as remaining or missing cells. Fifty OHCs of each row in the middle part of each turn of the cochlea were counted (a total of 150 cells per turn). Guinea pigs were administered 5 mg/kg of cisplatin or 50 mg/kg of carboplatin intraperitoneally for three consecutive days.

In groups 1 and 2, in which both cochleae were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide (OsO_4) and observed by SEM, the percentages of damage of the OHC stereocilia were similar in each cochlear turn bilaterally. **In group 3**, the right cochleae were fixed in OsO_4 and observed by phase contrast microscopy as surface preparations. Left cochleae were submitted for SEM observation. Missing and grade 3 cells were observed at similar percentages in each row of each turn. **In group 4**, succinate dehydrogenase staining was performed in the right cochleae and observed by LM. The degree of damage in the right cochleae was compared with that of the left cochleae which was observed by SEM. On average, the mean numbers of missing cells and cells showing grade 3 damage were similar in each row of each turn.

From these similarities of evaluation of ototoxicity at LM and SEM levels, it was concluded that semiquantitative analysis by SEM only is appropriate for the assessment of ototoxicity.

Key Words: Guinea pig, inner ear, sensory hair cells, stereocilia, ototoxicity, cisplatin, carboplatin, semiquantitative analysis, scanning electron microscopy.

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Introduction

Quantitative analysis for evaluating the ototoxicity of drugs such as aminoglycoside antibiotics (AGs) has been attempted in several ways. Observation of sections of outer hair cells (OHCs) by light microscopy (LM) and transmission electron microscopy (TEM) are useful for qualitative analysis. By these techniques, stereocilia and intracellular structure of OHCs can be observed simultaneously. Furthermore, the degree of damage in each turn of the cochlea can be roughly evaluated. Quantitative analysis, however, is very difficult to perform since these techniques require much more time and effort.

A technique called "surface preparation" was popularized by Engström *et al.* (1964) which made it possible to perform quantitative analysis at the LM level, and evaluation of ototoxicity of AGs was attempted (Hawkins and Engström, 1964; Engström and Kohonen, 1965; Brummett and Fox, 1982). Thirty to fifty OHCs and inner hair cells (IHCs) in each row were observed after osmium tetroxide (OsO_4) fixation, and counted as remaining or missing cells. These data were expressed as a cochleogram. By this technique, the stereocilia and the surface view of the cuticular plate were observed. The presence of cell bodies was also observed by optical sectioning. Many of the reports concerning ototoxicities of AGs have involved semiquantitative analysis in which a part of each turn of the cochlea is analyzed. If all hair cells were counted, the cochleograms represent quantitative analysis; it has been shown that these quantitative data were in agreement with functional data (Aran *et al.*, 1982; Lenoire *et al.*, 1983). The ototoxicities of the platinum anticancer drugs, cisplatin (CDDP) and carboplatin (CBDCA), were analyzed by this technique (Fleishman *et al.*, 1975; Schweitzer *et al.*, 1986; Taudy *et al.*, 1992; Saito and Aran, 1994). The disadvantage of this method, however, is the difficulty in evaluating whether the remaining cells were alive. To circumvent this problem, some histochemical stains were used, for example, succinate dehydrogenase (SDH), which is an enzyme of the respiratory system in mitochondria; living

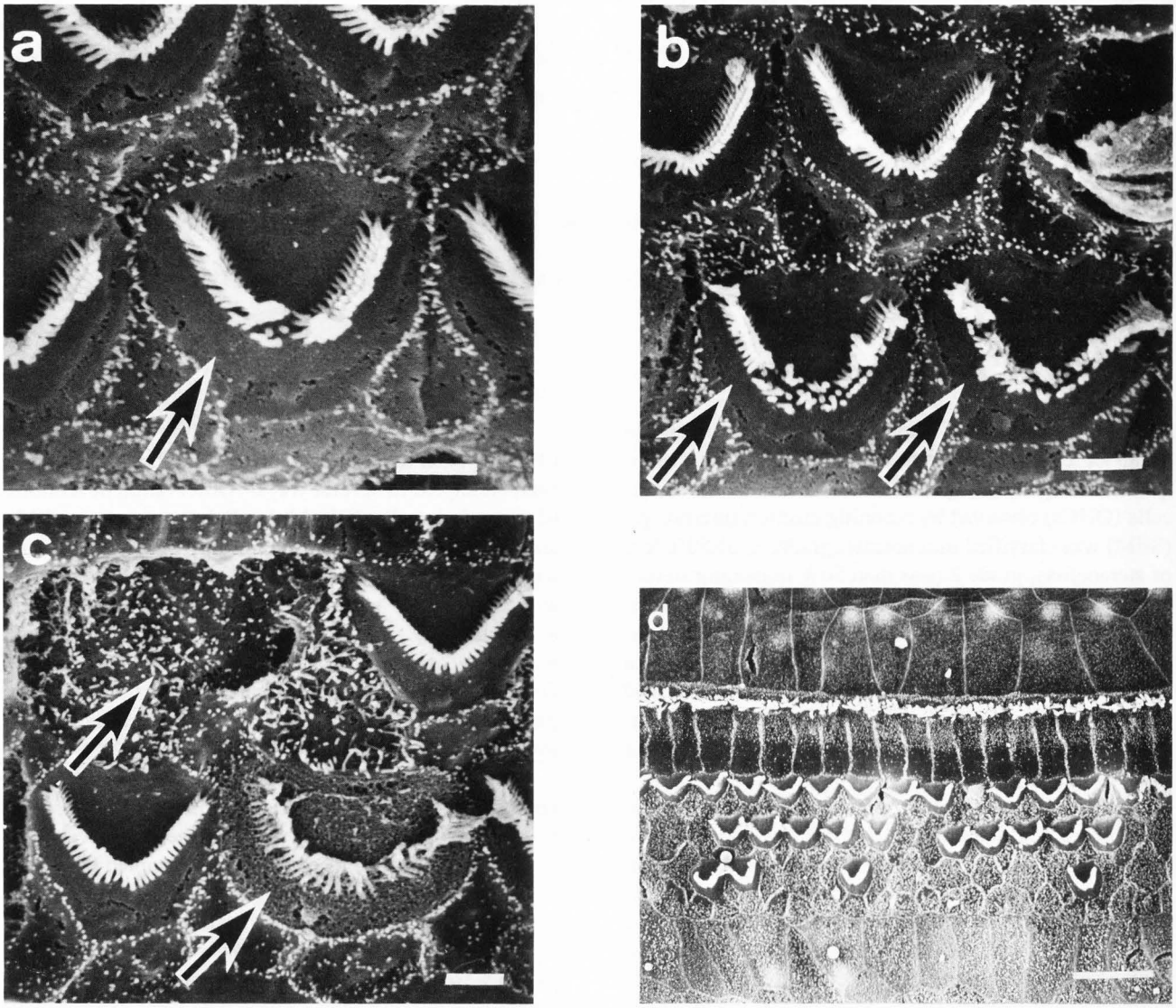

Figure 1. Scanning electron micrographs of the surface of the organ of Corti treated with 5 mg/kg of cisplatin for three consecutive days (**a**, **b** and **c**) and 50 mg/kg of carboplatin for three consecutive days (**d**). (**a**) Grade 1 outer hair cell (arrow). Stereocilia were missing in 10% to 50% of cell. Bar = 1 μm. (**b**) Grade 2 outer hair cells (arrows). Stereocilia remained in less than 50% of cell. Bar = 1 μm. (**c**) Grade 3 outer hair cell (arrows). Stereocilia had collapsed and the cuticular plate showed a rough surface (right lower corner). The OHC in left upper corner was replaced by a supporting cell. Bar = 1 μm. (**d**) Most outer hair cells were assessed as normal or grade 3. Grade 1 and 2 cells were rare in the basal cochlear turn. Bar = 10 μm.

OHCs and IHCs are specifically stained by this agent (Akiyoshi and Sato, 1967). Semiquantitative analysis of ototoxicity of CBDCA using this stain has been reported previously (Saito *et al.*, 1989b).

Recently, attempts at evaluating ototoxicity by observing the surface of the organ of Corti by scanning electron microscopy (SEM) have been reported (Laurell and Bagger-Sjöbäck, 1991b; Fernández-Cervilla *et al.*, 1993). Damage to the stereocilia of hair cells were divided into three or four grades in these studies. However, it is not known if observation of only the stereocilia is appropriate for the semiquantitative analysis of ototoxicity because there is a possibility that the damage of the stereocilia is not always consistent with that of the cell body. There have been no reports of a comparison between SEM analysis and conventional semiquantitative analysis by LM.

The aim of the present study was to compare semiquantitative analysis of ototoxicity by SEM with analysis by LM, and to determine whether the evaluation of oto-

toxicity by SEM is appropriate. For evaluation of ototoxicity at the LM level, OsO_4 fixation and SDH staining were used in the cochlea of guinea pigs treated with CDDP or CBDCA.

Materials and Methods

Albino guinea pigs (350 to 400 g) with good acoustic pinna reflexes were used.

Drug treatment

CDDP (Randa® Inj., Nippon Kayaku Co., Japan) and CBDCA solutions (Paraplatin Injection, Bristol-Myers Squib K.K., Japan) were administered to guinea pigs divided into 4 groups. Groups 1 and 3 consisted of 4 and 6 guinea pigs, respectively, treated with 5 mg/kg of CDDP for three consecutive days. Groups 2 and 4 consisted of 6 and 14 guinea pigs, respectively, treated with 50 mg/kg of CBDCA for three consecutive days. All drugs were administered intraperitoneally. The doses for administration were determined based on acute LD_{50} (Saito *et al.*, 1989a).

Observation of the surface of organ of Corti by SEM

Two or three days after the final drug administration, the guinea pigs were sacrificed, and their auditory bullae were removed. Both cochleae of groups 1 and 2 were fixed by perfusion of 2.5% glutaraldehyde (GA), and postfixed in 1% OsO_4. The left cochleae of Groups 3 and 4 were fixed as above for observation by SEM (Hitachi S-400). In the middle part of each turn of the cochlea (5 to 6 mm, 10 to 11 mm, 13 to 14 mm, 16 to 17 mm from the round window membrane), 50 OHCs in each row (total, 150 OHCs) were examined. A semiquantitative analysis of cochlear surface pathology was undertaken according to a four-grade scale established by Fredelius *et al.* (1987) and Laurell and Bagger-Sjöbäck (1991b): Normal: OHC with normal stereocilia; Grade 1: OHC with 10% to 50% damage or loss of stereocilia (Fig. 1a); Grade 2: OHC with less than 50% stereocilia remaining (Fig. 1b); Grade 3: rupture of the cuticular plate and missing hair cells. The OHCs, in which the cuticular plate was degenerating with or without disappearance of stereocilia (Fig. 1c) or those which were pushed toward the endolymphatic space by supporting cells, were counted as grade 3.

Thus, damage of OHCs was compared on both sides in groups 1 and 2.

Observation of hair cells by light microscopy

The right cochleae of group 3 were fixed by perfusion of 1% OsO_4. The strips of the organ of Corti from four turns of the cochlea were removed in 70% alcohol by the surface preparation technique (Engström *et al.*, 1964), and mounted on microscope slides. Then, the

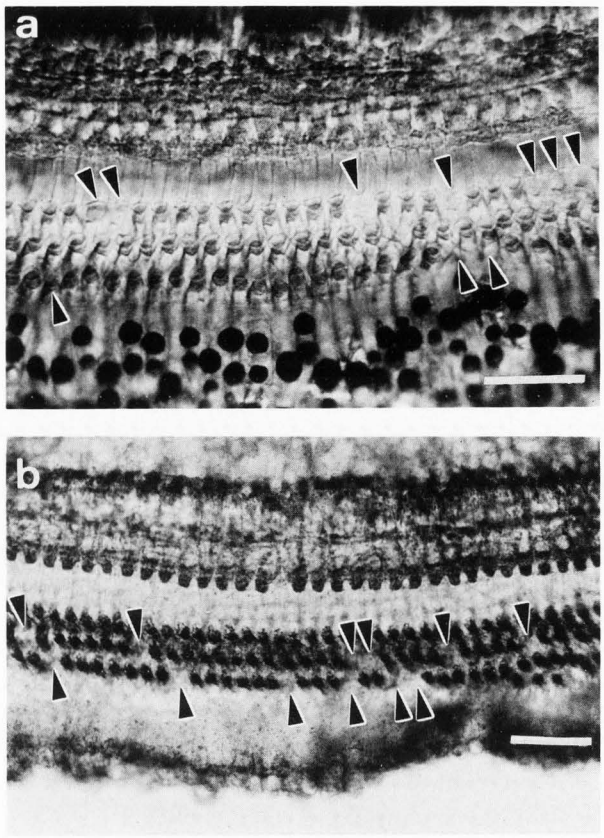

Figure 2. Light micrographs of the organ of Corti after surface preparation. (**a**) Surface view of the basal turn in the organ of Corti fixed with OsO_4 (Group 1). The cuticular plates and hair bundles can be seen. Some of the outer hair cells were missing in the first and third rows (arrowheads). Bar = 50 μm. (**b**) Three rows of outer hair cells and a row of inner hair cells were specifically stained by SDH (Group 4). Some of the outer hair cells were missing in the second and third rows (arrowheads). Bar = 50 μm.

OHCs were examined under a phase contrast microscope and classified into remaining and missing cells as shown in Figure 2a (Hawkins and Engström, 1964) in the same part of each turn of the cochlea as used for SEM examination. At first, the OHCs were observed at the level of the surface of the cuticular plate. If the surface showed an "X" or "Y" shape, this indicated that the OHC was replaced by a supporting cell. If the OHC was not replaced by a supporting cell, optical sectioning was performed. The OHC was counted as remaining when the cell body was stained.

The right cochleae of group 4 were prepared for SDH staining: the cochleae were perfused with Nachlas solution (a ratio of 1:1:2 mixture of 0.2 M sodium succinate, 0.2 M phosphate buffer solution at pH 7.6, and 0.1% nitro blue tetrazolium), immersed in the same

Table 1. Mean frequencies and standard deviations of the outer hair cells divided into four grades by scanning electron microscopy in each row and turn in guinea pigs treated with 5 mg/kg of cisplatin for three consecutive days (Group 1; n = 4). T1: basal turn, T2: second turn, T3: third turn, T4: apical turn, OHC 1: first row of the outer hair cell, OHC 2: second row, OHC 3: third row.

		Normal		Grade 1		Grade 2		Grade 3	
		Right	Left	Right	Left	Right	Left	Right	Left
T1	OHC 1	69.0±15.5	67.0±23.5	0.5± 1.0	1.0± 2.0	0	0	30.5±16.2	32.0±22.4
	OHC 2	63.5±30.5	57.5±31.5	0.5± 1.0	1.0± 1.2	0.5± 1.0	1.0± 1.2	35.5±29.6	40.5±30.4
	OHC 3	63.5±36.5	63.0±37.2	0	0	0	0.5± 1.0	36.5±36.5	36.5±36.4
T2	OHC 1	73.0±30.0	68.0±34.5	0.5± 1.0	0	0.5± 1.0	0.5± 1.0	26.0±29.6	31.5±33.5
	OHC 2	81.0±31.5	72.0±40.2	0.5± 1.0	1.0± 2.0	1.0± 2.0	4.0± 8.0	17.5±28.4	23.0±30.5
	OHC 3	88.5±11.5	87.0±17.0	7.0± 8.2	3.5± 7.0	0.5± 1.0	1.5± 2.0	4.0± 5.6	6.5± 9.5
T3	OHC 1	93.0±10.0	99.0± 1.0	0.5± 1.0	0	0.5± 1.0	0.5± 1.0	6.0±10.8	0.5± 1.0
	OHC 2	99.0± 1.2	97.0± 1.2	0.5± 1.0	2.0± 2.4	0	0	0.5± 1.0	1.0± 1.2
	OHC 3	74.0±17.0	74.5± 8.0	13.0±11.4	4.5± 6.5	7.5±10.4	5.5± 7.2	5.5± 4.5	15.5±11.5
T4	OHC 1	97.5± 2.0	97.5± 3.0	0	0	0.5± 1.0	0	2.0± 1.6	2.5± 3.0
	OHC 2	89.0±12.5	91.0± 9.5	4.5± 9.0	2.0± 2.8	3.0± 3.8	1.0± 2.0	3.5± 5.8	6.0± 7.5
	OHC 3	67.0±15.8	62.5±19.5	3.5± 2.0	7.5± 9.0	1.5± 2.0	1.5± 2.0	28.0±12.5	28.5±19.2

Table 2. Mean frequencies and standard deviations of the outer hair cells in guinea pigs treated with 50 mg/kg of carboplatin for three consecutive days (Group 2; n = 6). The cochleae were observed by scanning electron microscopy on both sides. T1-T4 are same as defined in Table 1.

		Normal		Grade 1		Grade 2		Grade 3	
		Right	Left	Right	Left	Right	Left	Right	Left
T1	OHC 1	96.0± 3.5	95.3± 7.6	0.3± 0.8	0.3± 0.8	0	0	3.7± 3.7	4.3± 7.8
	OHC 2	88.0±14.2	88.3±11.0	0.3± 0.8	2.0± 2.6	0	0	11.7±14.2	9.6±11.0
	OHC 3	67.0±25.2	62.7±20.5	2.7± 4.6	6.3± 6.3	0	0.3± 0.8	30.3±23.4	30.7±20.6
T2	OHC 1	90.3± 7.2	90.7±12.2	1.3± 3.2	0	0	0	8.0± 5.6	9.3±12.2
	OHC 2	68.0±19.8	69.0±23.6	5.0± 8.4	2.0± 4.0	0.7± 1.6	1.0± 2.4	26.3±12.0	28.0±22.7
	OHC 3	82.7± 9.2	84.0± 7.4	9.7± 9.4	5.0± 6.0	0.3± 0.8	1.3± 1.6	7.3± 6.6	9.7± 5.2
T3	OHC 1	92.3± 9.6	84.7±29.8	1.0± 1.0	0.7± 1.0	0	0.3± 0.8	6.7±10.0	14.3±29.2
	OHC 2	86.7± 8.4	87.7± 9.6	6.0± 2.8	8.7± 8.4	0.3± 0.8	1.0± 1.6	7.0± 7.4	2.7± 2.0
	OHC 3	75.6± 7.5	77.7±15.2	14.7± 6.8	11.0± 7.4	2.0± 3.0	3.0± 3.2	7.7± 6.2	8.3± 5.8
T4	OHC 1	90.7±10.6	94.7± 5.8	5.3± 7.6	1.0± 1.6	0	0	4.0± 5.6	4.3± 6.0
	OHC 2	75.0±17.2	82.7± 9.8	8.7± 8.0	5.3± 5.3	2.7± 2.4	2.7± 4.8	13.7±16.0	9.3± 4.8
	OHC 3	61.0±16.0	55.7±20.6	7.3± 7.2	3.7± 5.6	1.7± 2.4	1.0± 1.6	30.0±19.6	39.7±22.5

solution for 15 minutes at 37°C, and fixed in 10% formaldehyde followed by surface preparation of the strips of the organ of Corti (Akiyoshi and Sato, 1967). By examination under LM, the OHCs were divided into the same two groups as in group 3: remaining cells and missing cells (Fig. 2b).

The results of semiquantitative analysis of the right cochleae of groups 3 and 4 were compared with those of the left cochleae examined by SEM in the same animals.

Statistical analysis

Analysis of the significance of differences was calculated by Student's *t*-test.

Results

For ease of comparison, the experimental data for group 1 are summarized in Table 1, for group 2 in Table 2, for group 3 in Tables 3 and 4, and for group 4 in Tables 5 and 6.

Table 3. Individual data from group 3 animals (n = 6; No. 1-6). Outer hair cells of right ears after fixation with OsO_4 were assessed by phase contrast microscopy as remaining (Rem.) or missing (Mis.), and those of left ears assessed by scanning electron microscopy as normal (Nor.), grade 1 (Gr.1), grade 2 (Gr.2) or grade 3 (Gr.3).

No.1 Right ear

		Rem.	Mis.
T1	OHC 1	98	2
	OHC 2	98	2
	OHC 3	90	10
T2	OHC 1	100	
	OHC 2	100	
	OHC 3	96	4
T3	OHC 1	100	
	OHC 2	100	
	OHC 3	98	2
T4	OHC 1	98	2
	OHC 2	100	
	OHC 3	70	30

Left ear

		Nor.	Gr.1	Gr.2	Gr.3
T1	OHC 1	98			2
	OHC 2	94		2	4
	OHC 3	98			2
T2	OHC 1	96			4
	OHC 2	98			2
	OHC 3	98		2	
T3	OHC 1	100			
	OHC 2	100			
	OHC 3	92	4		4
T4	OHC 1	100			
	OHC 2	86	8		6
	OHC 3	88			12

No.2 Right ear

		Rem.	Mis.
T1	OHC 1	78	22
	OHC 2	86	14
	OHC 3	90	10
T2	OHC 1	94	6
	OHC 2	98	2
	OHC 3	98	2
T3	OHC 1	96	4
	OHC 2	98	2
	OHC 3	92	8
T4	OHC 1	100	
	OHC 2	94	6
	OHC 3	92	8

Left ear

		Nor.	Gr.1	Gr.2	Gr.3
T1	OHC 1	70			30
	OHC 2	86		2	12
	OHC 3	88		2	10
T2	OHC 1	90			10
	OHC 2	98			2
	OHC 3	94			6
T3	OHC 1	100			
	OHC 2	100			
	OHC 3	96			4
T4	OHC 1	98			2
	OHC 2	86	10		4
	OHC 3	84	10		6

No.3 Right ear

		Rem.	Mis.
T1	OHC 1	96	4
	OHC 2	100	
	OHC 3	98	2
T2	OHC 1	94	6
	OHC 2	96	4
	OHC 3	98	2
T3	OHC 1	98	2
	OHC 2	98	2
	OHC 3	96	4
T4	OHC 1	92	8
	OHC 2	86	14
	OHC 3	88	12

Left ear

		Nor.	Gr.1	Gr.2	Gr.3
T1	OHC 1	92			8
	OHC 2	100			
	OHC 3	94			6
T2	OHC 1	96			4
	OHC 2	100			
	OHC 3	98			2
T3	OHC 1	94			6
	OHC 2	96	4		
	OHC 3	90	6	2	2
T4	OHC 1	98			2
	OHC 2	50	48		2
	OHC 3	46	42		12

No.4 Right ear

		Rem.	Mis.
T1	OHC 1	74	26
	OHC 2	94	6
	OHC 3	82	18
T2	OHC 1	92	8
	OHC 2	100	
	OHC 3	98	2
T3	OHC 1	98	2
	OHC 2	96	4
	OHC 3	92	8
T4	OHC 1	94	6
	OHC 2	98	2
	OHC 3	88	12

Left ear

		Nor.	Gr.1	Gr.2	Gr.3
T1	OHC 1	72			28
	OHC 2	84			16
	OHC 3	86			14
T2	OHC 1	88			12
	OHC 2	94			6
	OHC 3	100			
T3	OHC 1	94			6
	OHC 2	94	4		2
	OHC 3	68	26		6
T4	OHC 1	98	2		
	OHC 2	100			
	OHC 3	56	28		16

No.5 Right ear

		Rem.	Mis.
T1	OHC 1	88	12
	OHC 2	98	2
	OHC 3	92	8
T2	OHC 1	98	2
	OHC 2	100	
	OHC 3	94	6
T3	OHC 1	100	
	OHC 2	98	2
	OHC 3	90	10
T4	OHC 1	96	4
	OHC 2	92	8
	OHC 3	76	24

Left ear

		Nor.	Gr.1	Gr.2	Gr.3
T1	OHC 1	84			16
	OHC 2	98			2
	OHC 3	96	2		2
T2	OHC 1	100			
	OHC 2	98			2
	OHC 3	94	2		4
T3	OHC 1	100			
	OHC 2	94			6
	OHC 3	82	2	2	14
T4	OHC 1	98			2
	OHC 2	90			10
	OHC 3	76	2		22

No.6 Right ear

		Rem.	Mis.
T1	OHC 1	62	38
	OHC 2	76	24
	OHC 3	84	16
T2	OHC 1	50	50
	OHC 2	90	10
	OHC 3	100	
T3	OHC 1	94	6
	OHC 2	98	2
	OHC 3	90	10
T4	OHC 1	80	20
	OHC 2	92	8
	OHC 3	72	28

Left ear

		Nor.	Gr.1	Gr.2	Gr.3
T1	OHC 1	22	2	6	70
	OHC 2	46	4		50
	OHC 3	44	4		52
T2	OHC 1	52			48
	OHC 2	88			12
	OHC 3	100			
T3	OHC 1	86			14
	OHC 2	98			2
	OHC 3	92			8
T4	OHC 1	94			6
	OHC 2	92	6		2
	OHC 3	86	12		2

Table 4. Mean frequencies and standard deviations of the outer hair cells assessed by phase contrast microscopy after fixation with OsO_4 (right ear) and by scanning electron microscopy (left ear). Guinea pigs were treated with 5 mg/kg of cisplatin for three consecutive days (Group 3; n = 6). Remaining: remaining cells, Missing: missing cells.

Right ear

		Remaining	Missing
T1	OHC 1	82.7±13.8	17.3±13.8
	OHC 2	92.0± 9.2	8.0± 9.2
	OHC 3	89.3± 5.8	10.7± 5.8
T2	OHC 1	88.0±18.8	12.0±18.8
	OHC 2	97.3± 4.0	2.7± 4.0
	OHC 3	97.3± 2.0	2.7± 2.0
T3	OHC 1	97.7± 2.4	2.3± 2.3
	OHC 2	98.0± 1.2	2.0± 1.2
	OHC 3	93.0± 3.2	7.0± 3.2
T4	OHC 1	93.3± 7.2	6.7± 7.2
	OHC 2	93.7± 5.0	6.3± 5.0
	OHC 3	81.0± 9.4	19.0± 9.4

Left ear

		Normal	Grade 1	Grade 2	Grade 3
T1	OHC 1	73.0±27.2	0.3± 0.8	1.0± 2.4	25.7±24.4
	OHC 2	84.7±20.0	0.7± 1.6	0.7± 1.0	14.0±18.6
	OHC 3	84.3±20.2	1.0± 1.6	0.3± 0.8	14.3±19.0
T2	OHC 1	87.0±17.5	0	0	13.0±17.5
	OHC 2	92.7±12.2	0	0	4.0± 4.4
	OHC 3	97.3± 2.8	0.3± 0.8	0.3± 0.8	2.4± 2.4
T3	OHC 1	95.7± 5.6	0	0	4.3± 5.7
	OHC 2	97.0± 2.8	1.3± 2.0	0	1.7± 2.4
	OHC 3	86.7±10.2	6.3± 9.9	0.7± 1.0	6.3± 4.2
T4	OHC 1	97.7± 2.0	0.3± 0.8	0	2.0± 2.2
	OHC 2	84.0±17.5	12.0±18.2	0	4.0± 3.6
	OHC 3	72.7±17.5	15.7±16.2	0	11.7± 7.0

Table 6. Mean frequencies and standard deviations of the outer hair cells assessed by light microscopy after SDH staining (right ear) and by scanning electron microscopy (left ear). Guinea pigs were treated with 50 mg/kg of carboplatin for three consecutive days (Group 4; n = 7). There was a significant difference (*$p < 0.05$) only in the comparison between remaining OHCs and those with normal stereocilia in OHC3 of the third turn. Remaining: remaining cells, Missing: missing cells.

Right ear

		Remaining	Missing
T1	OHC 1	91.7±10.8	8.3±10.7
	OHC 2	76.6±26.2	23.4±26.2
	OHC 3	62.9±22.0	37.1±22.0
T2	OHC 1	88.9± 9.4	11.1± 9.4
	OHC 2	79.1±16.8	20.9±16.8
	OHC 3	75.1±25.2	24.9±25.1
T3	OHC 1	98.6± 2.2	1.4± 2.2
	OHC 2	96.3± 5.4	3.7± 5.4
	OHC 3	93.7± 6.2	6.3± 6.2
T4	OHC 1	98.6± 2.5	1.4± 2.6
	OHC 2	90.9±11.2	9.1±11.2
	OHC 3	90.0±12.8	10.0±12.8

Left ear

		Normal	Grade 1	Grade 2	Grade 3
T1	OHC 1	96.0± 2.4	0	0.3± 0.8	3.7± 2.6
	OHC 2	81.7±14.2	0	0	18.3±14.2
	OHC 3	66.3±19.2	0.9± 2.2	0	32.9±20.0
T2	OHC 1	89.7± 9.6	2.6± 3.6	0.3± 0.8	7.4± 9.4
	OHC 2	71.4±16.8	2.0± 4.4	0.3± 0.8	26.3±17.6
	OHC 3	77.7±24.0	9.1±11.4	2.9± 6.0	10.3±16.4
T3	OHC 1	91.7±17.6	2.3± 6.0	4.3±11.4	1.7± 1.4
	OHC 2	88.0±13.8	7.7± 9.4	1.1± 1.6	3.1± 3.3
	OHC 3	*76.0±17.2	8.3±12.8	4.0± 6.6	11.7± 9.8
T4	OHC 1	98.9± 2.2	0	0	1.1± 2.1
	OHC 2	85.4±22.0	11.1±22.6	0.3± 0.8	3.1± 1.6
	OHC 3	80.0±25.2	11.4±21.2	0.3± 0.8	8.3± 7.4

Pattern of alteration of stereocilia

CDDP treatment group The damage to the stereocilia of OHCs in groups 1 and 3 involved mainly the basal turn, followed in a descending fashion by the second and third turns. There was no damage to the IHCs. In the basal turn of group 1, the stereocilia of three rows of OHCs showed grade 3 damage in 30% to 40% of the cells. In the basal turn of group 3, the first row of OHCs was most affected. In the second turn of groups 1 and 3, damage to the stereocilia involved essentially the first row, followed in descending order by the second and third rows of OHCs. On the other hand, the OHCs in the third row were most affected in the third and apical turns (Tables 1 and 4). In the degeneration process, the stereocilia became disarrayed and a decrease in their number was observed. At a later stage, loss of hair cells and their replacement by expansion of supporting cell phalanges were observed.

CBDCA treatment group In group 2, the damage of the OHCs involved essentially the basal and second

No.1 Right ear

		Rem.	Mis.
T1	OHC 1	68	32
	OHC 2	20	80
	OHC 3	36	64
T2	OHC 1	96	4
	OHC 2	56	44
	OHC 3	38	62
T3	OHC 1	98	2
	OHC 2	98	2
	OHC 3	96	4
T4	OHC 1	100	
	OHC 2	98	2
	OHC 3	98	2

Left ear

		Nor.	Gr.1	Gr.2	Gr.3
T1	OHC 1	94			6
	OHC 2	64			36
	OHC 3	32			68
T2	OHC 1	92	2	2	4
	OHC 2	46			54
	OHC 3	28	10		62
T3	OHC 1	100			
	OHC 2	96			4
	OHC 3	94			6
T4	OHC 1	100			
	OHC 2	94			6
	OHC 3	98			2

No.2 Right ear

		Rem.	Mis.
T1	OHC 1	100	
	OHC 2	96	4
	OHC 3	94	6
T2	OHC 1	98	2
	OHC 2	90	10
	OHC 3	84	16
T3	OHC 1	100	
	OHC 2	100	
	OHC 3	100	
T4	OHC 1	100	
	OHC 2	84	16
	OHC 3	96	4

Left ear

		Nor.	Gr.1	Gr.2	Gr.3
T1	OHC 1	98			2
	OHC 2	94			6
	OHC 3	66			34
T2	OHC 1	98			2
	OHC 2	78			22
	OHC 3	94			6
T3	OHC 1	98			2
	OHC 2	100			
	OHC 3	98	2		
T4	OHC 1	100			
	OHC 2	98			2
	OHC 3	100			

No.3 Right ear

		Rem.	Mis.
T1	OHC 1	94	6
	OHC 2	82	18
	OHC 3	48	52
T2	OHC 1	84	16
	OHC 2	76	24
	OHC 3	92	8
T3	OHC 1	98	2
	OHC 2	98	2
	OHC 3	100	
T4	OHC 1	96	4
	OHC 2	98	2
	OHC 3	98	2

Left ear

		Nor.	Gr.1	Gr.2	Gr.3
T1	OHC 1	92			8
	OHC 2	76			24
	OHC 3	56			44
T2	OHC 1	82	4		14
	OHC 2	90			10
	OHC 3	100			
T3	OHC 1	52	16	30	2
	OHC 2	98			2
	OHC 3	88			12
T4	OHC 1	98			2
	OHC 2	98			2
	OHC 3	98			2

No.4 Right ear

		Rem.	Mis.
T1	OHC 1	98	2
	OHC 2	90	10
	OHC 3	72	28
T2	OHC 1	96	4
	OHC 2	90	10
	OHC 3	100	
T3	OHC 1	100	
	OHC 2	98	2
	OHC 3	90	10
T4	OHC 1	100	
	OHC 2	96	4
	OHC 3	96	4

Left ear

		Nor.	Gr.1	Gr.2	Gr.3
T1	OHC 1	98			2
	OHC 2	100			
	OHC 3	92			8
T2	OHC 1	98			2
	OHC 2	88			12
	OHC 3	88			12
T3	OHC 1	98			2
	OHC 2	90	6	2	2
	OHC 3	70	2	18	10
T4	OHC 1	100			
	OHC 2	96			4
	OHC 3	92			8

No.5 Right ear

		Rem.	Mis.
T1	OHC 1	92	8
	OHC 2	76	24
	OHC 3	52	48
T2	OHC 1	80	20
	OHC 2	92	8
	OHC 3	40	60
T3	OHC 1	100	
	OHC 2	98	2
	OHC 3	94	6
T4	OHC 1	94	6
	OHC 2	68	32
	OHC 3	62	38

Left ear

		Nor.	Gr.1	Gr.2	Gr.3
T1	OHC 1	96			4
	OHC 2	76			24
	OHC 3	74			26
T2	OHC 1	96			4
	OHC 2	72			28
	OHC 3	70	30		
T3	OHC 1	98			2
	OHC 2	90	10		
	OHC 3	66		2	32
T4	OHC 1	100			
	OHC 2	96		2	2
	OHC 3	78	2		20

No.6 Right ear

		Rem.	Mis.
T1	OHC 1	96	4
	OHC 2	78	22
	OHC 3	50	50
T2	OHC 1	74	26
	OHC 2	56	44
	OHC 3	84	16
T3	OHC 1	94	6
	OHC 2	84	16
	OHC 3	82	18
T4	OHC 1	100	
	OHC 2	98	2
	OHC 3	92	8

Left ear

		Nor.	Gr.1	Gr.2	Gr.3
T1	OHC 1	96			4
	OHC 2	68			32
	OHC 3	64			36
T2	OHC 1	72	2		26
	OHC 2	52	2		46
	OHC 3	86	6		8
T3	OHC 1	96			4
	OHC 2	60	26	4	10
	OHC 3	54	28	6	12
T4	OHC 1	100			
	OHC 2	38	60		2
	OHC 3	32	56		12

No.7 Right ear

		Rem.	Mis.
T1	OHC 1	94	6
	OHC 2	94	6
	OHC 3	88	12
T2	OHC 1	100	
	OHC 2	92	8
	OHC 3	98	2
T3	OHC 1	100	
	OHC 2	98	2
	OHC 3	94	6
T4	OHC 1	100	
	OHC 2	94	6
	OHC 3	88	12

Left ear

		Nor.	Gr.1	Gr.2	Gr.3
T1	OHC 1	98		2	
	OHC 2	94			6
	OHC 3	80	6		14
T2	OHC 1	90	10		
	OHC 2	74	12	2	12
	OHC 3	78	18	4	
T3	OHC 1	100			
	OHC 2	82	12	2	4
	OHC 3	62	26	2	10
T4	OHC 1	94			6
	OHC 2	78	18		4
	OHC 3	62	22	2	14

Table 5. Individual data from group 4 animals (n = 7; No. 1-7). Outer hair cells of right ears after SDH staining were assessed by light microscopy as remaining (Rem.) or missing (Mis.), and those of left ears assessed by scanning electron microscopy as normal (Nor.), grade 1 (Gr.1), grade 2 (Gr.2) or grade 3 (Gr.3).

turns, followed by the third and apical turns of the cochlea. The degeneration of stereocilia tended to be more severe in the third row of the basal and apical turns and the second row of the second turn, whereas the stereocilia in the first row of the third turn was most affected (Table 2). In general, CBDCA had less ototoxicity than CDDP.

Only half of the animals in group 4 showed hair cell damage in this study. Therefore, 7 of 14 animals were used for analysis. In group 4, damage of the OHCs was observed mainly in the basal and second turns, as with group 2. The pattern of stereocilial damage in the basal, second and apical turns was the same as in group 2, whereas the third row was most affected in the third turn (Table 6).

The stereocilia of the IHCs remained intact in both groups.

Semiquantitative analysis by SEM

Most OHCs could be divided into normal or grade 3 (Fig. 1d). The percentages of grades 1 and 2, on the other hand, were low. In groups 1 (CDDP) and 2 (CBDCA), in which both ears were observed by SEM, damage in the right and left ears in each animal were similar, although there was considerable inter-individual variation. Furthermore, mean percentages of damage were similar on both sides (Tables 1 and 2), and therefore, the damage to the stereocilia on both sides was similar when the regions where the observation was performed were the same on both sides in each turn of the cochlea.

Comparative analysis between SEM and LM observation

CDDP treatment group In group 3, the right ear was examined by phase contrast microscopy and the left ear was examined by SEM. In a comparison between the percentages of missing and grade 3 cells, the difference between sides was not significant in any animal (Table 3). Although the mean percentage of damage to the stereocilia in the basal turn was slightly higher than that of missing cells, the tendency for damage to be most severe in the innermost row was similar. The mean percentages of damage in the second turn, on the other hand, were similar on both sides (Table 4). The rates of grades 1 and 2 were low, especially in the basal and second turns. Therefore, the difference between the percentages of missing cells and those of grade 1, 2, and 3 cells did not increase except in the apical turn. From these results, it was shown that assessment of damage of the OHCs by conventional OsO_4 fixation and of damage of the stereocilia by SEM examination were similar.

CBDCA treatment group In group 4, there was considerable inter-individual variation and a slight difference between right and left ears (Table 5). In comparing mean percentages of missing and grade 3 cells, the former was slightly higher than the latter in the basal and second turns. However, the tendency for damage to be higher in outer rows of OHCs (OHC 2 and 3) was similar on both sides. In the third and apical turns, on the other hand, there was no significant difference between missing and grade 3 cells (Table 6).

Discussion

Examination of the organ of Corti by SEM is needed to observe the upper surface of the hair cells and supporting cells. For assessment of ototoxicity, if the sensory hair cell had disappeared and was replaced by a supporting cell, it was easy to assess it as missing. For SEM examination, preparation of the specimen was relatively easy, and the surface of all turns of the cochlea could be observed in a single specimen. Therefore, this procedure might be used as in assessment of damage to the inner ear. Since semiquantitative analysis of the surface of organ of Corti by SEM after acoustic overstimulation was performed by Fredelius *et al.* (1987), assessment of ototoxicity using the same method has been attempted by other investigators. Ototoxic assessment of CDDP was performed by Laurell and Bagger-Sjöbäck (1991b) using the four-grade system used in this study. Fernández-Cervilla *et al.* (1993) divided the morphological changes of the stereocilia into three grades for semiquantitative analysis of CDDP ototoxicity: complete orthotypic pattern, partially denuded pattern, and totally denuded pattern. These reports revealed the same tendency of damage of OHCs as conventional assessment by LM. However, changes in the cell body have not been investigated. Therefore, it must be clarified whether the results of assessment by SEM agree with those by conventional methods.

In the present study, the right cochleae were examined by LM after OsO_4 fixation or SDH staining and compared with the left cochleae of the same animal examined by SEM. OsO_4 fixation has been established to determine the presence or absence of the cell body (Engström *et al.*, 1964) . Positive staining by OsO_4, however, does not indicate that the cell is alive. Therefore, SDH staining was used in this study to distinguish whether the stained cell was living; SDH is one of the enzymes of respiratory system in mitochondria and living hair cells were stained specifically by this agent. In other groups, the degree of damage in both ears was evaluated only by SEM to assess the difference between right and left ears. As a result, damage to the stereocilia on both sides were similar individually and on average. It has been reported previously that the condition

of both ears fixed by OsO_4 was usually symmetrical after AGs intoxication (Aran *et al.*, 1982). By comparing the right ear after fixation by OsO_4 or SDH staining and the left ear examined by SEM in this study, the difference between sides was not so remarkable in each animal. Furthermore, the average amount of damage on both sides was similar: that is to say, the rate of missing cells as shown by LM examination was in close agreement with the percentage of grade 3 cells by SEM examination, and damage to the cell body and that to the stereocilia were correlated.

Damage to the OHCs treated with CDDP or CBDCA involved mainly the basal and second turns. With CDDP, damage began from the innermost row in the basal and second turns. These results are comparable to the findings reported previously by many authors (Fleishman *et al.*, 1975; Estrem *et al.*, 1981; Nakai *et al.*, 1982; Konishi *et al.*, 1983; Böheim and Bichler, 1985; Marco-Algarra *et al.*, 1985; Schweitzer *et al.*, 1986; Saito *et al.*, 1989a,b; Laurell and Bagger-Sjöbäck, 1991a,b; Fernández-Cervilla *et al.*, 1993). As a consequence of the development of damage, all three rows were affected in group 1. Although hair cell damage was often found in the apical turns of normal control animals (Lenoire *et al.*, 1983; Schweitzer *et al.*, 1986), the rate of damage became higher in some animals after treatment with CDDP and CBDCA, as shown in this study as well as by Fernández-Cervilla *et al.* (1993). On the other hand, some authors have reported no ototoxic effect of CBDCA (Schweitzer *et al.*, 1986; Ohtani *et al.*, 1989; Taudy *et al.*, 1992). However, the dosage of 50 mg/kg for three days that was used in this study caused damage in all turns of the cochlea (Saito *et al.*, 1989b). The nature of damage, however, was quite different from that caused by CDDP: OHC 3 in the basal turn, OHC 2 in the second turn and OHC 3 in the third and apical turns showed a tendency to be affected. This phenomenon was similar to that with application of AGs (Theopold, 1977; Aran *et al.*, 1982; Lenoire *et al.*, 1983), although the mechanism is unclear. IHCs were not affected by CBDCA in guinea pigs, whereas toxicity to the IHCs was reported in chinchillas (Wake *et al.*, 1993).

In early morphological changes of the stereocilia induced by CDDP, the membrane roughness of the stereocilia became exaggerated and the number of lateral cross-links was reduced from that seen by high resolution SEM (Comis *et al.*, 1986; Osborne and Comis, 1990). Thereafter, the hair bundles became disarrayed, and a decrease in number or fusion of the stereocilia was seen. The four-grade scale used in this study involved only the degree of disappearance of stereocilia. Therefore, disarray of the stereocilia was not considered in grading. The rates of grades 1 and 2 were lower than those reported previously by Laurell and Bagger-Sjöbäck (1991b). However, it is very difficult to judge whether grade 1 and 2 cells are living or dead. From the TEM findings after AG intoxication reported by Forge (1985), grade 1 and 2 cells seem to have been degenerating. Therefore, it is possible that these cells would show a positive reaction to OsO_4 and SDH staining. For simultaneous observation of the stereocilia and intracellular structure, TEM observation will be needed. From our previous report using TEM, the stereocilia and cuticular plate were shown to have degenerated as a consequence of the development of cell damage after treatment with CDDP (Saito and Aran, 1994). As shown in OHCs treated with AGs (Forge, 1985), hair cells then ruptured in the lateral membrane. Apical fragments were retained in the reticular lamina and became surrounded by the expanded supporting cells. The debris of the apical fragments were released into the endolymphatic space.

The grading system employed in this study has been used previously for the assessment of damage of the stereocilia after acoustic overstimulation (Fredelius *et al.*, 1987). In the case of acoustic trauma, damage to the stereocilia precedes cell body damage; grades 1 and 2 occur relatively soon after exposure, whereas grades 3 and 4 develop over time. Our assessment was performed two or three days after the final treatment with CDDP. As a result, the majority of cells were classified as normal or grade 3, and the rate of grades 1 and 2 was low in this study. This is different from the results of a previous study by Laurell and Bagger-Sjöbäck (1991b) in which the rate of grade 2 OHCs treated with CDDP was higher. This difference suggests the possibility that the standards of judgement for grades 2 and 3 were different. We judged the cells whose stereocilia were completely degenerated, but in which the stereocilia debris remained on the surface of the degenerating cuticular plate as grade 3. Therefore, it was considered appropriate to assess the cells of grade 3 as missing while normal and grades 1 and 2 stereocilia damage were assessed as remaining.

Finally, the number of cells classed as grade 3 by SEM examination was similar to that of missing cells as determined by LM observation, and it was obvious morphologically that the grade 3 cells were dead. Semiquantitative analysis by SEM is suitable for assessing the ototoxicity of CDDP and CBDCA. However, it was very difficult to assess whether the grade 1 and 2 cells were living. Further investigation by TEM will be needed, in which the condition of the stereocilia and the structure of the cuticular plate and cell body should be thoroughly examined on the same cell. This may elevate the assessment of ototoxicity by SEM examination to a more accurate and reliable level.

References

Akiyoshi M, Sato K (1967). Histochemical demonstration of succinic dehydrogenase activity of the hair cells by means of intravital perfusion of the cochlea. Audiology Japan **10**, 48-56.

Aran JM, Erre JP, Guilhaume A, Aurouseau C (1982). The comparative ototoxicities of gentamicin, tobramycin and dibekacin in the guinea pig. Acta Otolaryngol (Stockh) Suppl **390**, 1-30.

Böheim K, Bichler E (1985). Cisplatin-induced ototoxicity: Audiometric findings and experimental cochlear pathology. Arch Otorhinolaryngol **242**, 1-6.

Brummet RE, Fox KE (1982). Studies of aminoglycoside ototoxicity in animal models. In: The aminoglycosides. Whelton A, Neu HC (eds.). Marcel Dekker, Inc. New York. pp. 419-437.

Comis SD, Rhys-Evans PH, Osborne MP, Pickles JO, Jeffries DJR, Pearse HAC (1986). Early morphological and chemical changes induced by cisplatin in the guinea pig organ of Corti. J Laryngol Otol **100**, 1375-1383.

Engström H, Ades HW, Hawkins JE (1964). Cytoarchitecture of the organ of Corti. Acta Otolaryngol (Stockh) Suppl **188**, 92-99.

Engström H, Kohonen A (1965). Cochlear damage from ototoxic antibiotics. Acta Otolaryngol (Stockh) **59**, 171-178.

Estrem SA, Babin RW, Ryu JH, Moore KC (1981). Cis-diammine dichloroplatinum(II) ototoxicity in the guinea pig. Otolaryngol Head Neck Surg **89**, 638-645.

Fernández-Cervilla F, Crespo PV, Ciges M, Campos A (1993). Early morphofunctional alterations induced by cisplatin in the cochlea. ORL **55**, 337-340.

Fleishman RW, Stadnicki SW, Ethier MF, Schaeppi U (1975). Ototoxicity of cis-dichlorodiammine platinum (II) in the guinea pig. Toxicol Appl Pharmacol **33**, 320-332.

Forge A (1985). Outer hair cell loss and supporting cell expansion following chronic gentamicin treatment. Hear Res **19**, 171-182.

Fredelius L, Johansson B, Bagger-Sjöbäck D, Wersäll J (1987). Qualitative and quantitative changes in the guinea pig organ of Corti after pure tone acoustic overstimulation. Hear Res **30**, 157-168.

Hawkins JE, Engström H (1964). Effect of kanamycin on cochlear cytoarchitecture. Acta Otolaryngol (Stockh) Suppl **188**, 100-107.

Konishi T, Gupta BH, Prazma AJ (1983). Ototoxicity of cis-dichlorodiammine platinum (II) in guinea pigs. Am J Otolaryngol **4**, 18-26.

Laurell G, Bagger-Sjöbäck D (1991a). Degeneration of the organ of Corti following intravenous administration of cisplatin. Acta Otolaryngol (Stockh) **111**, 891-898.

Laurell G, Bagger-Sjöbäck D (1991b). Dose-dependent inner ear changes after I.V. administration of cisplatin. J Otolaryngol **20**, 158-167.

Lenoire M, Marot M, Uziel A (1983). Comparative ototoxicity of four aminoglycoside antibiotics during the critical period of cochlear development in the rat. Acta Otolaryngol (Stockh) Suppl **405**, 1-16.

Marco-Algarra J, Basterra J, Marco J (1985). Cis-diammine dichloro platinum ototoxicity. Acta Otolaryngol (Stockh) **99**, 343-347.

Nakai Y, Konishi K, Chang KC, Ohashi K, Morisaki N (1982). Ototoxicity of the anticancer drug cisplatin. Acta Otolaryngol (Stockh) **93**, 227-232.

Ohtani I, Anzai T, Aikawa T, Okamura H (1989). Toxicity of carboplatin vs. cisplatin. Otologica (Kyoto) Suppl **32**, 49-55.

Osborne MP, Comis SD (1990). High resolution scanning electron microscopy of stereocilia in the cochlea of normal, postmortem, and drug-treated guinea pigs. J Electron Microsc Tech **15**, 245-260.

Saito T, Sakashita T, Honda N, Manabe Y, Wakui S, Saito H (1989a). Comparison of ototoxicity among four kinds of anticancer platinum drugs in guinea pigs. Ear Res Jpn **20**, 50-55.

Saito T, Saito H, Saito K, Wakui S, Manabe Y, Tsuda G (1989b). Ototoxicity of carboplatin in guinea pigs. Auris Nasus Larynx (Tokyo) **16**, 13-21.

Saito T, Aran JM (1994). Comparative ototoxicity of cisplatin during acute and chronic treatment. ORL **56**, 315-320.

Schweitzer VG, Rarey KE, Dolan DF, Abrams G (1986). Ototoxicity of cisplatin vs. platinum analogs CBDCA (JM-8) and CHIP (JM-9). Otolaryngol Head Neck Surg **94**, 458-470.

Taudy M, Syka J, Popelár J, Úlehlová (1992). Carboplatin and cisplatin ototoxicity in guinea pigs. Audiology **31**, 293-299.

Theopold HM (1977). Comparative surface studies of ototoxic effects of various aminoglycoside antibiotics on the organ of Corti in the guinea pig. Acta Otolaryngol (Stockh) **84**, 57-64.

Wake M, Takeno S, Ibrahim D, Harrison R, Mount R (1993). Carboplatin ototoxicity: An animal model. J Laryngol Otol **107**, 585-589.

Discussion with Reviewers

A. Campos: Postfixation with osmium tetroxide, as some authors have indicated, destroys cross-links and change the appearance of stereocilia. How do you think this may have affected your findings?

Authors: In our present study, we mainly focused to the decreased number of stereocilia. As reported by

Comis *et al.*(1986) and Osborne and Comis(1990), the lateral cross-links were reduced in number or absent in early morphological changes induced by cisplatin. However, the numbers of stereocilia were not reduced by postfixation using osmium tetroxide in non-treated animals. Therefore, we consider that postfixation by osmium tetroxide does not affect the number of stereocilia even after treatment with cisplatin.

A. Campos: Is there any type of statistical analysis that could be applied to your findings to back up your conclusions?
Y. Harada: Ideally, statistical analysis would be helpful to potentiate authors' point of view described in **Semiquantitative analysis by SEM**; "therefore, the damage to the cilia on both sides was similar...".
Authors: Student *t*-test (unpaired) was performed. In groups 1 and 2, OHCs with normal stereocilia and those of grade 3 were compared in each row and turn on both sides. In groups 3 and 4, statistical analysis was performed between remaining OHCs and OHCs with normal stereocilia, and between missing cells and grade 3 cells in each row and turn. There were no significant differences in any of our analytical groups except OHC3 of third turn in group 4.

S.D. Comis: I feel that a word of caution about the use of albino animals would be appropriate here in view of known differences in the response between albino and pigmented animals to certain ototoxic agents [see for example the article by GR Bock and KP Steel: Use of albino animals for auditory research. Hear Res (1984) **13**, 201-202].
Authors: From our recent study concerning the ototoxicity induced by cisplatin, albino guinea pigs were more susceptible than pigmented ones. In our present study, however, we compared ototoxicities of cisplatin and carboplatin using albino animals, as it is inexpensive to obtain albino guinea pigs in our country because of the establishment of a Hartley strain for animal experiments.

Y. Harada: Have the authors ever tried to prepare the same cochlea for LM surface preparation and SEM examination at the same time? If this is feasible with minimal artificial effects, it will give a direct and shortcut comparison of the two different preparation methods which are focused in this article.
A. Forge: It is quite easily possible to examine tissue that has been examined by light microscopy of surface features by SEM after additional processing especially when tissue is fixed by both GA and Os. Would this not have made it easier to prepare the light microscopy and SEM results?
Authors: In this study, we have not examined the same cochlea by LM and SEM. As two reviewers point out, it is necessary to examine the same cochlea at both LM and SEM levels. This will be the next step in this study.

A. Forge: What is the extent of damage caused? For this, the normal procedure would be to wait until the damage had reached a stable condition (3-4 weeks after the end of treatment) then count the number of missing hair cells.
Authors: We also consider that fixation should wait until the damage reaches a stable state. From our previous study, the dose of 5 mg/kg of CDDP or 50 mg/kg of CBDCA for three consecutive days caused certain damage to OHCs. However, the guinea pigs can live only for several days after these treatments because of their strong toxicity. We decided, therefore, the time of sacrifice of animals as two or three days after treatment.

A. Forge: There are several reports that show that following drug-induced damage to hair cells, the surface features of those cells are not a good indicator of whether or not a cell is damaged. There is some evidence that noise does affect stereocilia directly; changes in stereocilia after drug toxicity may only be a secondary reflection of the fact that a cell is damaged and may not occur at all. It may not be valid to use analysis developed for noise trauma with ototoxicity.
Authors: We had doubts at first about the reports of Laurell and Bagger-Sjöbäck (1991b) and Fernández-Cervilla *et al.* (1993) concerning the assessment of ototoxicity of CDDP using only SEM because of the possibility that the stereocilia might appear normal when the hair cell body showed obvious damage. From the similarities of evaluation of ototoxicity by conventional methods (i.e., assessment at LM level) and SEM, it was concluded that analysis only by SEM is not inappropriate for assessment of ototoxicity. Further investigation will be needed, however, to determine whether or not the grading system used in noise trauma can be applied to ototoxicity.