

THE APOPTOTIC PROCESS IN THE AUDITORY HAIR CELLS OF THE MOUSE AFTER 6 HOURS OF NEOMYCIN TREATMENT

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REZUMAT

Objective: Evaluarea răspunsului apoptotic în primele 6 ore după expunerea la neomicină a celulelor auditive senzoriale din organul lui Corti. **Material și metode:** Celulele auditive prelevate de la șoareci C57BL6 au fost cultivate in vitro în prezența (lotul tratat cu neomicină, n = 59 cohlei) sau absența neomicinei (lotul de control, n = 31 cohlei). Celulele apoptotice au fost evaluate după 6 ore de expunere la neomicină, prin evidențierea caspazelor active 8, 9 sau 3 din citoplasmă și a nucleilor marcați cu propidium iodid. Celulele au fost cuantificate statistic folosind media și deviația standard. Diferențele dintre medii au fost testate cu ajutorul testului Student t ($p < 0,05$). **Rezultate:** Comparatia statistică între diferitele tipuri de caspaze (caspaza 3, 8 și 9), media și dispersia în cadrul grupului celular, ne-a permis detectia primei etape apoptotice care are loc în citoplasmă. Comparatia grupului celular unde propidium iodid s-a acumulat în nucleu, cu celulele în care erau prezente atât marcajele pentru caspaze, cât și propidium iodid, ne-a permis detectia inițierii și a dezvoltării etapei secundare a apoptozei, etapă în care nucleul este de asemenea afectat. **Concluzii:** Neomicina inițiază după 6 ore de incubație procesul apoptotic în celulele auditive, determinând activarea caspazelor citoplasmice.

Cuvinte cheie: apoptoză, celule senzoriale auditive, neomicină

ABSTRACT

Objectives: To study the apoptotic response of auditory hair cells in the organ of Corti within the first 6 hours after exposure to neomycin. **Material and methods:** Auditory hair cells harvested on the postnatal day 7 from the cochleas of C57BL6 wild type mice were cultured in vitro in the presence (neomycin group, n = 59 cochleas) or absence of neomycin (control group, n = 31 cochleas). Apoptotic cells were evaluated within 6 hours of incubation for the expression of active caspases (8, 9 or 3) in the cytoplasm and counterstained using propidium iodide to highlight apoptotic nuclei. Cells were statistically quantified using average and standard deviation. The differences between the averages were tested with the help of Student t test ($p < 0.05$). **Results:** The statistic comparison between the different caspase types (caspase 3, 8 and 9), the average and dispersion inside the cell groups, permitted us to detect the first step of apoptosis taking place in the cytoplasm. The comparison of the cell group where propidium iodide accumulated in the nucleus with the cell group where both the caspase and propidium iodide appeared, permitted us to detect the initiation and development of the second step of apoptosis, the one where the nucleus has already been affected. **Conclusions:** Neomycin is initiating, right after 6 hours of incubation, the process of apoptosis in auditory hair cells, determining the activation of cytoplasm caspases.

Key Words: Apoptosis, auditory hair cells, neomycin

INTRODUCTION

Many in vivo and in vitro studies have suggested that aminoglycosides determine hair cell death in the organ of Corti.

Morphological evidence suggest that hair cells loss in response to aminoglycoside treatment occurs via

apoptosis.^{1,2} At least two types of cellular degeneration are recognized in tissues, necrosis and apoptosis. The cytologic features of these are quite distinct.³

In recent years, specific intracellular proteases belonging to the caspase family have surfaced as crucial effectors of apoptosis.^{4,5} Caspases are expressed as pro-enzymes and are activated by upstream stimuli, determining the beginning of the apoptotic process. There are data that indicate that undamaged mouse vestibular hair cells express pro-caspases 3, 7, 8 and 9, and that activation of caspase 9 may be crucial for neomycin-induced apoptosis.^{6,7}

There are two major apoptotic pathways according to the type of pro-caspase which is activated. Activation of initiator pro-caspase 8 results from signaling via cell surface death receptors such as Fas and TNFR1.^{5,8,9} On the other hand, activation of pro-caspase 9 is

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dependent primarily on mitochondrial signalling pathways regulated by members of the Bcl-2 gene family. Caspase 9, a non-receptor mediated caspase, is activated when cytochrome c is released from mitochondria into the cytoplasm. Cytochrome c binds to Apaf-1 leading to apoptosome-mediated activation of caspase 9. The translocation of cytochrome c to the cytoplasm appears to be due to the release of intermembrane proteins (SIMP) through the opening of the mitochondrial permeability transition pore (MPTP) and/or the rupture of the mitochondrial outer membrane.¹⁰ Recent studies suggest that cytochrome c and caspase 9 play critical roles in aminoglycoside-induced hair cell death.¹¹⁻¹⁴ Cunningham et al. suggest that only inhibitors of caspase 9 suppressed caspase 3 activation and prevented neomycin-induced hair cell death.⁶

Laura Zöllner and H. Löwenheim (2005) from Tübingen Hearing Research center, have registered the apoptotic changes that occur in the Organ of Corti under neomycin influence after 24 and 12 hours of incubating the inner ears in bioreactors, in a culture medium with neomycin. We had the mission to establish the cytotoxic effect of neomycin upon auditory hair cells after 6 hours of *in vitro* contact of the cochleas with the antibiotic.

OBJECTIVES

Our research had the following purposes:

1. To establish if after a six hour contact of the cochleas with 1 mM neomycin we can find death between sensory hair cells in the organ of Corti;
2. To establish if cell death is realized through necrosis or apoptosis;
3. To know if cells in the three cochlear segments (basal, middle and apical) are equally sensitive to the cytotoxic effect of neomycin.

In order to obtain correct responses to these questions, we organized a number of experiments on cochleas cultivated *in vitro* under same conditions, having as variable factor the presence or absence of neomycin from the culture medium. In the following chapter we will describe in detail the working techniques.

MATERIAL AND METHODS

The study was performed in the Department of Molecular Otolaryngology, of the Hearing Research Center, Marie Curie training site in Tübingen, Germany. Our work is included in a research program which has the

purpose to understand the modifications which are produced in the structure of the hearing cells under the influence of ototoxic aminoglycosides treatment.

Organisation of experiments

In order to answer the question, whether neomycin can induce hair cell death in the organ of Corti, we harvested cochleas (n = 90) from seven days old mice, which we split in two groups. One group of cochleas was named neomycin group and was cultivated for 6 hours in a microgravity rotator bioreactor in the presence of 1 mM neomycin. In order to analyze caspase 3 we treated 21 cochleas with neomycin. For highlighting caspase 8, 16 cochleas were treated with neomycin. For caspase 9, 22 cochleas were treated with neomycin. Another group of cochleas was cultivated under the same conditions but in the absence of neomycin (caspase 3 with 10 control cochleas; caspase 8 with 7 control cochleas and caspase 9 with 14 control cochleas). In order to obtain a good response to the question if hair cell death due to neomycin is realized by necrosis or apoptosis, the two cochlear groups were sacrificed, fixed and specifically stained, after six hours of cultivation in bioreactors. We performed specific staining in order to evidence active caspases in hair cells (3, 8 and 9) as well as to evidence the level of nuclear degeneration by propidium iodide staining. Both the presence of active caspases in cellular cytoplasm as well as the appearance of propidium iodide in the nucleus, are indicative that cell death occurs through apoptosis and not necrosis. The quantification of hair cell death due to both apoptosis and necrosis was performed with the help of phalloidin staining, a specific marker for F-actin in the stereocilia.

Cochlear culture preparation

The study was performed in the Tübingen Hearing Research Center (Marie Curie Program).

We obtained organotypic cultures of the organ of Corti (n = 45) from postnatal day seven (p7) C57BL6 mouse pups. The mice were sacrificed by decapitation. Organ explantation was approved by the Committee for Animal Experiments of the Regional Council (Regierungspräsidium Tübingen). The temporal bones were dissected in HEPES- buffered saline with Hank's salts (HHBSS) at pH 7.3 under sterile conditions. The dissection period of one ear lasted around five minutes. In order to allow diffusion of cell culture medium to the sensory epithelium, the bony labyrinths are widely opened to allow fluid exchange to the perilymphatic space. The extension of the opening of the scala tympani perilymphatic space beginning at

the round window and prepared along the basal turn in apical direction. The thin bony shell covering the scala tympani perilymphatic space is further removed along 270° of the basal turn. The next step was to open the scala vestibuli perilymphatic space achieved by removing the bony cap covering the apical turn of the cochlea. Care was taken not to injure the endolymphatic space in all preparations. The Corti organs were incubated in rotatory cell culture microgravity systems with or without 1 mM neomycin (RCCS TM, Synthecon Inc. Houston) for six hours at 37°C and 5% CO₂/95% air, in 55 ml culture medium. The rotation speed was set to 30 rotations/minute and the position of the rotation vessels was vertical. Cultivation was carried forward under continuous rotation. The culture medium consisted of Neurobasal™ A medium (Gibco) supplemented with B27 supplement (Gibco), 1M Hepes buffer (Gibco), 0.5 mM L-glutamine (Gibco) and 15 units/ml of penicillin. The ears were splitted into control and neomycin probes. In order to destroy the hair cells we added 1 mM neomycin (SIGMA).

Treatment of cultures and micro dissection

After the initial culture period of 6 hours, the cochleas were removed from the rotation vessels, and incubated for 40 minutes at 37°C in 48-wellplates (Corning Incorporated, NY) in the caspase inhibitor solution, depending on the caspase we wanted to check: caspase 8, 9 or 3. In order to visualize the localization of the caspases, we used cell permeable fluorochrominhibitors (FLICA) from CHEMICON caspase kits, for each caspase: caspase 3 - FAM-DEVD-FMK (APT 403); caspase 8 - FAM-LETD-FMK (APT 428) and for caspase 9 - FAM-LEHD-FMK (APT 429), Chemicon. The lyophilized FLICA reagent was mixed with 50 µl DMSO (SIGMA) and then, immediately before use, 1:5 diluted in PBS (pH = 7.4) in order to obtain a 30x solution. All the procedures were done under light protection. After that, the cochleas were washed three times for 10 minutes in wash buffer (diluted 1:10 in distilled water). The next step was to stain the apoptotic nuclei with propidium iodid (CHEMICON)(4,5 µl) for 4 minutes on ice. The cochleas were then fixed with 50 µl fixing solution (CHEMICON) for 2 hours on ice. After that, we washed the cochleas twice in PBS and maintained them overnight in 1% PFA at 4°C. The next day, the cochleas were microdissected in PBS under a ZEISS microscope by entirely removing the bony labyrinth and the modiolus. The membranous cochlea was split in three parts: a basal, medial and apical part. From each segment we removed the Reissner and

tectorial membrane. The cochlea parts were then placed on Superfrost thin glass in Vectashield (Vector laboratories).

Fluorescence microscopy

Once the caspase inhibitors have entered the cell, they bind covalently on the active center of the caspase and the prosthetic chromophore group permits a green fluorescent light emission of 520 nm. Propidium iodid can be excited with a 488 line of an argon-ion laser and its absorption is maximum at 535 nm.

The organs were analyzed under a Confocal Laser Scanning Microscope (LSM) Pascal system (ZEISS) with a Neon-Helium-Laser (543 nm, Lasos) and an Argon-Laser (488 nm, Lasos). We used an LSM software (EMBL, Heidelberg) and a AxioCam MR c5 camera. The photos were taken with the 25-th objective under immersion oil. We measured the length of the entire Corti tunnel, the length of the caspase staining, the one of the propidium Iodid (PI) staining and the length of Caspase- PI staining. After that, we counted each stained hair cell (inner hair cells and the three rows of outer hair cells) for caspase (green), PI (red) and caspase- PI (yellow). Each curve needed a number of shots: the apical part 8 photos, the medial part 5-8 and the basal part 3-5.

Statistical analysis

The analysis of the data was performed with the help of Microsoft Excel program (Windows 2000). For the statistical analysis, we used Student T test and standard deviation in order to obtain a significance level. For T test, we took as significant the values $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.005$ (***). In general we performed 3-4 experiments x 14-16 Corti organs per each caspase. For caspase 9 we used 36 cochleas, from which 22 cochleas were treated with neomycin and 14 were controls. For caspase 8 we used 16 neomycin-treated cochleas and 7 controls. Caspase 3 experiments included 20 neomycin-treated cochleas and 10 controls. We analyzed the caspase expression pro mm of Corti organ (controls, neomycin), caspase expression depending on the segment (controls and neomycin) and caspase expression depending on the cell type (controls, neomycin).

RESULTS

1. Cytotoxic effect of neomycin upon sensorial hair cells in the organ of Corti

Cell death occurs in two ways. One is necrosis and the other is apoptosis. Apoptosis can be detected by the

presence of active caspases and propidium iodide in the cells. Hair cell death in the organ of Corti, irrespective of the cause, can be evidenced by the presence or absence of stereocillia with the help of phalloidin, a marker for F-actin in hair cell stereocillia.

Evidencing hair cell death due to both apoptosis and necrosis

In Figure 1 we expose the obtained results from our experiments regarding auditory cell death in general in cochleas treated and not treated with neomycin.

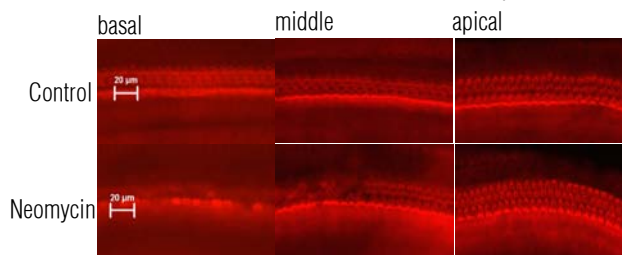


Figure 1. Neomycin-induced sensorial cell degeneration and cellular death 6 hours post-treatment. Phalloidin staining characteristic to F-actin in the stereocillia. After neomycin treatment we can observe the disappearance of stereocillia and the appearance of the characteristic scar.

From Figure 1, one can observe that after 6 hours of cochlear contact with neomycin, cellular death occurs only in the basal and middle cochlear segment. Cells which have lost their stereocillia and which present instead the specific scar are more frequent in cochleas treated with neomycin than in the control group. In the organs exposed we can observe a structural degradation of external auditory hair cells compared to internal auditory hair cells. In order to sustain this idea, we expose in Figure 2 the proportion of dead hair cells by apoptosis and necrosis in the three cochlear segments belonging to the control and neomycin group.

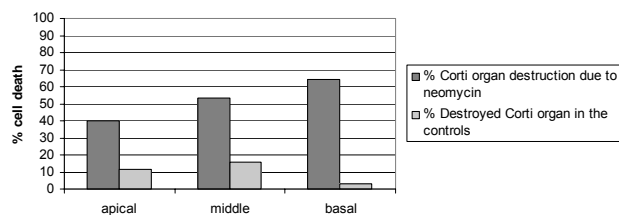


Figure 2. Neomycin-induced stereocilliary loss.

Data from Figure 2 suggest that neomycin induces stereocilliary loss first in the basal segment of the cochlea, followed by the middle segment and finally by the apical one. Hair cell death after 6 hours from the beginning of the treatment reaches in the basal segment more than 60% of the cochlear length, followed by 52% in the middle segment and 40% in the apical one. It is interesting to observe that a certain amount of

hair cells lose their stereocillia even outside neomycin treatment. This phenomenon seems to appear mostly in the middle segment and less in the basal one.

2. Apoptotic neomycin-induced hair cell death

Phalloidin staining is a marker for stereocilliary F-actin. Hair cell death due to neomycin can be seen clearly with the help of this method that demonstrates the disappearance of the stereocillia and the appearance of the characteristic scar. In order to estimate the number of hair cells which have died only because of apoptosis and not necrosis, we have stained the cochleas in order to evidence active cytoplasm caspases 3, 8 and 9 and nuclear propidium iodide. Obtained results are shown in Figure 3.

Because apoptotic hair cell death due to neomycin was similar in all analyzed slides marked for caspase 3, 8 and 9, we will present only the results for caspase 8 and counterstained with propidium iodide.

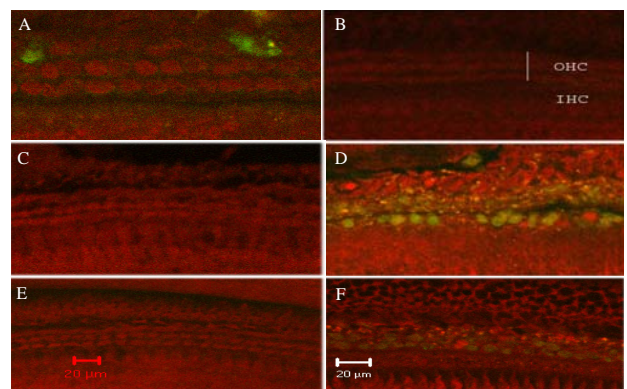


Figure 3. Activation of caspase 8 (green) and PI nuclear staining (red). Nuclei of apoptotic cells appear bright red and round and the cytoplasm stained green. We can also remark the presence of apoptotic bodies stained yellow. IHC: outer hair cells; IHC: inner hair cells. Left (A,C,E): controls without neomycin; right (B,D,F): after 6 hours neomycin treatment. After segments: apical (A,B), medial (C,D) and basal (E,F). Scale bar: 20 µm.

Figure 3 reveals the frequency of cells stained for caspase 3, 8 and 9 (green color of the cytoplasm) as well as with propidium iodide (red color of nuclei), from the in vitro cultivated cochleas, in the presence or absence of neomycin. From microscopic analysis of the two groups of cochleas we can observe that the frequency of cells stained green, with red nuclei, or green cytoplasm with red nuclei, present in the neomycin treated group, is superior to the one in the control group. The fact that the amplitude of caspase and propidium iodide stained cell frequency in cochleas treated with neomycin is similar to the amplitude of cell death evidenced with phalloidin, suggests that hair cell death in the organ of Corti is realized mostly by apoptosis and less by necrosis. (Fig. 2)

The fact that in the basal and middle segments of

the cochleas belonging to the neomycin group we can observe a larger number of cells stained for the three active caspases (3, 8 and 9) in the cytoplasm, compared to control cochleas, sustains the hypothesis that neomycin induces apoptosis in auditory hair cells.

3. Quantification of neomycin-induced apoptotic hair cell death

In order to establish if qualitative observations specified above are real, we tried to statistically quantify the apoptotic process. On this purpose we counted all cells with green cytoplasm, marking the presence of active cytoplasmic caspases 3, 8 and 9 in all cochleas belonging to the two groups (control and neomycin). Obtained data were then statistically processed obtaining the mean and standard deviation for each group and segment of cultivated cochlea. Results are presented in Figure 4.

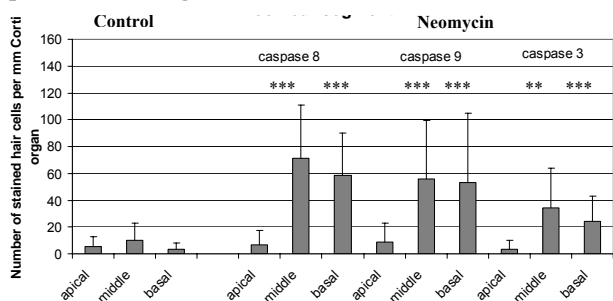


Figure 4. The average number of hair cells in the segments of the Corti organ marked for caspase 3, 8 and 9 after 6 hours of neomycin contact, compared to the stained cells in the control group.

Figure 4 shows as columns the average number of stained cells for each of the three active caspases (8, 9 and 3). The vertical line stands for average standard deviation. The significance of the differences between the average of apoptotic cells in the three cochlear segments from the neomycin treated group compared to the control one is symbolized with $p < 0.05$ or $p < 0.005$. The data from Figure 4 suggest that during the 6 hours of neomycin influence, many phenomena take place. These are the following:

1. As the average value of hair cells where we identified the three types of active caspases is significantly higher in the cochleas cultivated in the presence of neomycin compared to the control group, it has lead us to the conclusion that neomycin induces and sustains the apoptotic process of self destruction in auditory hair cells. The process starts to manifest itself after 6 hours of neomycin contact.

2. Simultaneous active caspase 8, 9 and 3 presence in cell cytoplasm (around 20/60 cells per mm Corti organ) shows that they participate together in this process and in a certain way in inducing and realizing

the apoptotic process in the organ of Corti

3. Neomycin ototoxic activity followed by apoptotic unleash at 6 hours from neomycin contact of the cochleas, are manifested only in the basal and middle segments of the Corti organ. Cells in the apical area of the Corti organ are almost not affected by the ototoxic neomycin effect.

4. High standard deviation values in all neomycin treated cochlear groups suggest that the sensitivity of hearing cells in the organ of Corti towards neomycin is very variable and different from one individual to another.

5. The fact that the average number of cells marked for active caspases is highest in the group marked for caspase 8 followed in a decreasing order by other caspases, suggests that there is a hierarchy in the participation of the three caspases in initiating and sustaining the apoptotic process.

4. Phases of the apoptotic process in auditory hair cells after 6 hours of neomycin treatment

While the apoptotic process can be identified at the beginning by evidencing cytoplasmic caspase 3, 8 and 9, the advancement of the process towards the nucleus, can be identified by propidium iodide nuclear incorporation and the red colour of the nucleus. In order to know if after 6 hours of neomycin treatment, apoptosis is at its onset or if the process has started much earlier, we have counted all red-coloured, round nuclei within the 3 experiments concerning caspase 3, 8 and 9 and processed them statistically. Results are shown in Figure 5.

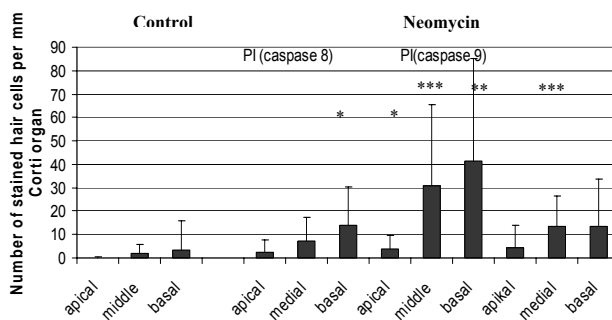


Figure 5. The average number of cells where apoptosis has involved also their nuclei in cochleas stained for caspase 3, 8 and 9, in the presence or absence of neomycin.

The average number of cells which have incorporated propidium iodide in their nuclei and their standard deviations, in the three cochlear groups stained for active caspases suggests that in auditory hair cells, at 6 hours from the contact with neomycin, the following processes take place:

1. The fact that auditory hair cell number where propidium iodide has accumulated is significantly

higher within the experiments concerning activated caspase 3, 8 and 9 compared to the control group, suggests that the apoptotic process doesn't start only at 6 hours after neomycin treatment, but probably much earlier.

2. The fact that the average number of cells which have incorporated propidium iodid in their nuclei (red nuclei) is significantly higher ($p < 0.005$), in cochleas treated with neomycin compared to the control group, is a solid argument which sustains the idea that neomycin initiates the process of apoptosis by activating the three procaspases. On their turn, these are further sustaining the process of nuclear destruction (propidium iodid penetrates the nucleus only if its membrane is affected).

3. Apoptosis is present also in hair cell nuclei, especially in the basal and middle cochlear segment and is almost absent in the apical segment.

4. Although the number of cells where apoptosis has reached the nucleus is higher in cochleas stained for caspase 9 compared to other experiments, the differences are not significant because of high standard deviation.

5. High deviation standard values suggest that hair cell sensitivity towards cytotoxic action of neomycin is very different from one individual to another because of genetic differences between each individual.

Apoptosis can be evidenced also at the beginning of nuclear destruction

Propidium iodide can penetrate the nuclear membrane only if its membrane is lesioned. The stage where the apoptotic process involves not only the cytoplasm but also the nucleus can be evidenced with the help of nuclear incorporation of propidium iodid together with cytoplasm staining for caspases. At this level cells appear coloured in green (for the cytoplasm) and red (for the nucleus). Our results concerning neomycin and control cochlear groups are shown in Figure 6.

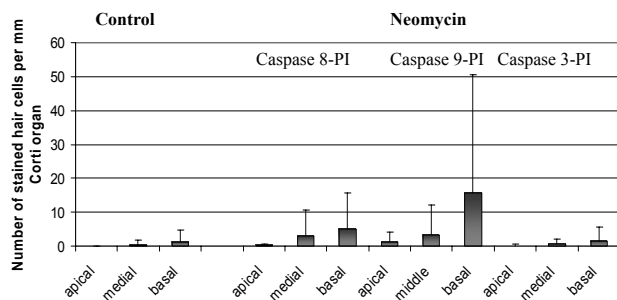


Figure 6. The average number of cells where apoptosis has reached both the cytoplasm and the nucleus (intermediary stage of apoptosis) in cochleas stained for caspase 3, 8 and 9, in the presence or absence of neomycin.

Data shown in Figure 6 reveal that in cochleas treated with neomycin and specifically stained for each of the three active cytoplasm caspases, we can also find enough cells where the apoptotic process is at the beginning in the nucleus. The number of such cells is much higher in the neomycin group compared to the controls. Because of the high standard deviations, the differences between the cochlear groups are not statistically significant. Therefore, we cannot draw any conclusion regarding this phenomenon which takes place in the two cochlear groups.

CONCLUSIONS

Experiments done on mice cochleas cultivated for 6 hours in the presence or absence of neomycin and specifically stained for apoptosis through cytoplasm presence of active caspase 8, 9 and 3 and nuclear propidium iodid presence allow us to draw the following conclusions:

1. Neomycin induces massive hair cell death in the organ of Corti after six hours of incubation with the cochleas.

2. Hair cell death in the organ of Corti is produced especially by apoptosis and less by necrosis (in dead cells active caspase 3, 8 and 9 are present in the cytoplasm and propidium iodid in the nucleus).

3. Initiation and finalizing of hair cell apoptosis due to neomycin take place only in the basal and middle segment of the Corti organ. Hair cells in the apical segment are almost not affected by ototoxic effect of neomycin.

4. Auditory cell sensitivity towards neomycin is very different from one individual to another.

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