

IMPACT OF GENTAMICIN ON ANTIOXIDANT ENZYMES ACTIVITY IN HEMn-DP CELLS

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Abstract: Aminoglycoside antibiotics, including gentamicin, are widely used clinically in treatment of bacterial infections. Unfortunately, their side effects, especially nephrotoxicity and ototoxicity remain a problem. It is known that aminoglycoside antibiotics bind well to melanin biopolymer, but the relation between their affinity to melanin and ototoxicity is not well documented. The aim of this work was to examine the impact of gentamicin on antioxidant enzymes activity in cultured dark pigmented normal human melanocytes (HEMn-DP). The WST-1 assay was used to detect gentamicin cytotoxic effect. The analyzed antibiotic induced concentration-dependent loss in melanocytes viability. The value of EC_{50} was found to be 7.5 mM. Significant changes in the cellular antioxidant enzymes: SOD, CAT and GPx were stated in melanocytes exposed to gentamicin, what may indicate the depletion of antioxidant defense system. It is concluded, that the results obtained *in vitro* may explain a potential role of melanocytes and melanin in the causative mechanisms of aminoglycosides ototoxic effects *in vivo*.

Keywords: gentamicin; melanocytes; superoxide dismutase; catalase, glutathione peroxidase

Gentamicin is a widely used aminoglycoside antibiotic, however the clinical usefulness of this drug is limited due to its ototoxicity and nephrotoxicity. Nephrotoxicity is reversible, but destruction of auditory system is irreversible, resulting in permanent hearing loss (1-5). Many studies have reported that gentamicin-induced ototoxicity is mediated by reactive oxygen species (ROS) (1, 6, 7). Overproduction of ROS triggers the signaling pathways of cellular apoptosis, resulting in inner ear damage. Several agents that scavenge ROS or block their formation (8-10) may be considered as protective substances that reduce ROS-induced ototoxicity (11, 12).

Melanin is a unique pigmented biopolymer synthesized by melanocytes in human and various animals species. In humans, melanin is found in the skin, eyes, brain and ear (13, 14). Many investigators have demonstrated the affinity of natural and synthetic melanins for various drugs by *in vivo* and *in vitro* studies. It is generally accepted that the ability of melanin-containing tissue to accumulate and retain these drugs is remarkable (15-17).

In view of the fact that melanin is an abundant constituent of the inner ear, it seems reasonable to

suspect that the specificity of gentamicin ototoxicity may result from its ability to form a complex with melanin. This phenomenon may contribute to the accumulation of the antibiotic in the inner ear and facilitate a toxic effect on surrounding cells.

Previously, we have documented that gentamicin, kanamycin, amikacin, neomycin, tobramycin and netilmicin (18-23) form stable complexes with model synthetic melanin *in vitro*. Our studies have also demonstrated that amikacin (24), kanamycin (25), netilmicin (26) and streptomycin (27) suppress melanin biosynthesis and cause depletion of antioxidant defense system in human light pigmented melanocytes (HEMa-LP).

To our knowledge, the effect of gentamicin on biochemical processes in melanocytes has never been reported. Therefore, we investigated the antioxidant enzymes activity in gentamicin-treated dark pigmented melanocytes HEMn-DP.

EXPERIMENTAL

Chemicals

Amphotericin B was purchased from Sigma-Aldrich Inc. (USA). Gentamicin and neomycin sul-

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fates were obtained from Amara, Poland. Penicillin was acquired from Polfa Tarchomin (Poland). Growth medium M-254 and human melanocytes growth supplement-2 (HMGS-2) were obtained from Cascade Biologics (UK). Trypsin/EDTA was obtained from Cytogen (Poland). Cell Proliferation Reagent WST-1 was purchased from Roche GmbH (Germany). The remaining chemicals were produced by POCH S.A. (Poland).

Cell culture

The normal human dark pigmented melanocytes (HEMn-DP, Cascade Biologics, UK) were grown according to the manufacturer's instruction. The cells were cultured in M-254 medium supplemented with HMGS-2, penicillin (100 U/mL), neomycin (10 µg/mL) and amphotericin B (0.25 µg/mL) at 37°C in 5% CO₂. All experiments were performed using cells in the passages 7-10.

Cell viability assay

The viability of melanocytes was evaluated by the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) colorimetric assay. WST-1 is a water-soluble tetrazolium salt, the rate of WST-1 cleavage by mitochondrial dehydrogenases correlates with the number of viable cells. In brief, 5000 cells per well were placed in a 96-well microplate in a supplemented M-254 growth medium and incubated at 37°C and 5% CO₂ for 48 h. Then, the medium was removed and cells were treated with gentamicin solutions in a concentration range from 0.01 to 10 mM. After 21 h incubation, 10 µL of WST-1 were added to 100 µL of culture medium in each well, and the incubation was continued for 3 h. The absorbance of the samples was measured at 440 nm with a reference wavelength of 650 nm, against the controls (the same cells but not treated with gentamicin) using a microplate reader UVM 340 (Biogenet, Poland). The controls were normalized to 100% for each assay and treatments were expressed as the percentage of the controls.

Superoxide dismutase (SOD) assay

Superoxide dismutase (SOD) activity was measured using an assay kit (Cayman, MI, USA) according to the manufacturer's instruction. This kit utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme needed to produce 50% dismutation of superoxide radical. SOD activity was expressed in U/mg protein.

Catalase (CAT) assay

Catalase (CAT) activity was measured using an assay kit (Cayman, MI, USA) according to the manufacturer's instruction. This kit utilizes the peroxidatic function of CAT for determination of enzyme activity. The method is based on the reaction of enzyme with methanol in the presence of an optimal concentration of H₂O₂. The formaldehyde produced is measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. One unit of CAT was defined as the amount of enzyme that causes the formation of 1.0 nM of formaldehyde per minute at 25°C. CAT activity was expressed in nM/min/mg protein.

Glutathione peroxidase (GPx) assay

Glutathione peroxidase (GPx) activity was measured using an assay kit (Cayman, MI, USA) according to the manufacturer's instruction. The measurement of GPx activity is based on the principle of a coupled reaction with glutathione reductase (GR). The oxidized glutathione (GSSG) formed after reduction of hydroperoxide by GPx is recycled to its reduced state by GR in the presence of NADPH. The oxidation of NADPH is accompanied by a decrease in absorbance at 340 nm. One unit of GPx was defined as the amount of enzyme that catalyzes the oxidation of 1 nM of NADPH per minute at 25°C. GPx activity was expressed in nM/min/mg protein.

Statistical analysis

In all experiments, the mean values of at least three separate experiments (n = 3) performed in triplicate ± standard error of the mean (SEM) were calculated. The results were analyzed statistically using GraphPad Prism 6.01 software. A value of p < 0.05 (*) or p < 0.005 (**), obtained with a Student's *t*-test by comparing the data with those for control (cells without gentamicin), was considered statistically significant.

RESULTS AND DISCUSSION

Although aminoglycosides induce ototoxicity and nephrotoxicity, they are still important antibiotics in current clinical practice and are widely used, especially in developing countries. Aminoglycosides exhibit broad-spectrum of antibacterial activity against enterococcal, mycobacterial, and especially multi-drug-resistant Gram-negative bacterial infections (2, 3). Additionally, aminoglycosides are commonly used in experimental ototoxicity models (28-30).

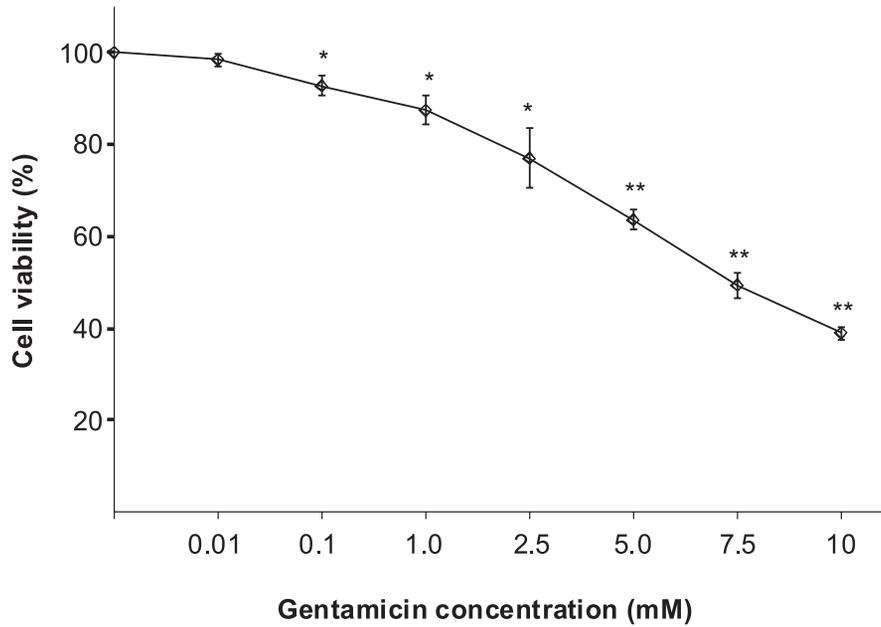


Figure 1. The effect of gentamicin on viability of melanocytes. Cells were treated with various concentrations of gentamicin (0.01-10 mM) and examined by WST-1 assay. Data are expressed as % of cell viability. The mean values \pm SEM from three independent experiments (n = 3) performed in triplicate are presented. * $p < 0.05$ vs. the control samples; ** $p < 0.005$ vs. the control samples

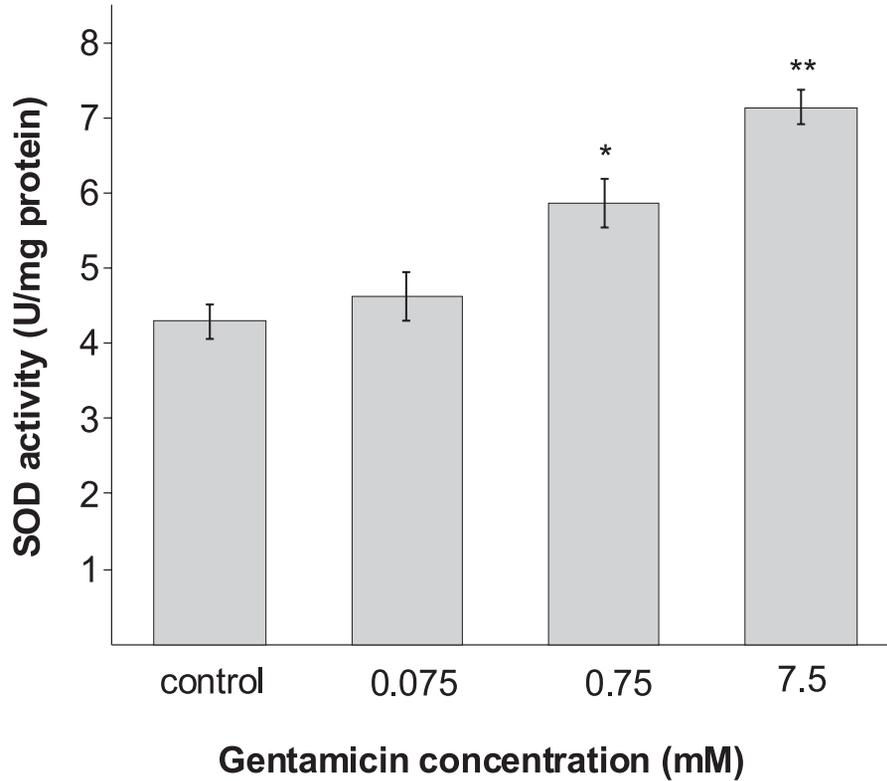


Figure 2. The superoxide dismutase (SOD) activity in HEMn-DP cell after 24 h incubation with 0.075, 0.75 or 7.5 mM of gentamicin. Data are the mean \pm SEM of at least three independent experiments (n = 3) performed in triplicate. * $p < 0.05$ vs. the control samples; ** $p < 0.005$ vs. the control samples

Aminoglycoside-induced ototoxicity progresses from high to low frequencies with an increase in dose or duration of treatment (2). It has been suggested that the relationship between the aminoglycoside treatment and pigmented tissue damage is important to understand the ototoxic process. Many drugs are known to be markedly accumulated and retained for a considerable time by melanin-containing tissues and the retention of these compounds is proportional to degree of pigmentation. The ability of melanins to bind different drugs and transition metal ions is probably of the greatest biological importance (15).

The aim of the present study was to investigate the effect of gentamicin on antioxidant defense system in HEMn-DP melanocytes. Human melanocytes develop from the neural crest, later becoming distributed in the epidermis, hair bulbs of the skin, the uveal tract, the retinal pigment epithelium, the inner ear, and the leptomeninges, which are collectively regarded as pigmented organs. In this study, we have used the culture of normal human melanocytes as an *in vitro* experimental model system.

Melanocytes were treated with gentamicin in a range of concentrations from 0.01 mM to 10 mM for 24 h (Fig. 1). The cell viability was determined by the WST-1 test assay. At a relative low antibiotic concentration (0.01 mM) the loss in cell viability was not statistically significant. Cells treated with 0.1, 1.0, 2.5, 5.0, 7.5 and 10 mM of gentamicin for 24 h lost about 7.3, 12.5, 23.0, 36.3, 50.6 and 61.1% in viability, respectively. The value of EC_{50} (i.e., the amount of a drug that produces loss in cell viability by 50%) was 7.5 mM.

The ototoxic effects of aminoglycosides have been linked to oxidative stress. Aminoglycosides such as gentamicin can react with iron to generate ROS within the inner ear, with permanent damage to hair cells and neurons (2, 28, 31). Overproduction of ROS was suggested as an initial step in triggering apoptotic pathways, resulting in cell death due to aminoglycoside-induced ototoxicity. Methods of blocking ROS in the cochlea under *in vitro* or *in vivo* aminoglycoside exposure have been analyzed in many studies (31, 32). Although the ototoxicity caused by aminoglycosides is well documented, the molecular mechanism have not yet been precisely determined.

To explain the effect of the tested antibiotic on ROS metabolism, the activities of the antioxidative enzymes: SOD, CAT and GPx in melanocytes were estimated.

SOD converts superoxide radical anion ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2). In the presence of

reduced transition metal ions (e.g., Fe^{2+} and Cu^+), hydrogen peroxide can be converted into highly reactive hydroxyl radical (OH^{\bullet}). Alternatively, hydrogen peroxide may be converted into water by the enzymes catalase or glutathione peroxidase (33).

Human melanocytes HEMn-DP were exposed to gentamicin in concentrations of 0.075, 0.75 or 7.5 mM (EC_{50}) for 24 h. The first enzyme measured was the SOD, i.e., the enzyme which catalyzes the formation of hydrogen peroxide from superoxide anion. Gentamicin enhanced the SOD activity in a concentration-dependent manner (Fig. 2). Treatment of cells with 0.75 and 7.5 mM of gentamicin, increased the SOD activity by 37 and 66%, respectively, as compared with the controls. CAT and GPx work together to catalyze the breakdown of hydrogen peroxide, produced by SOD, to water. The intracellular CAT activity was significantly decreased by 24% for cells treated with gentamicin in EC_{50} concentration (7.5 mM). (Fig. 3). After gentamicin treatment in concentrations of 0.075 and 0.75 mM, no significant changes in cellular CAT activity were determined in comparison with the control cells (Fig. 3). The activity of GPx increased by 26% for melanocytes treated with gentamicin in concentration of 0.75 mM and decreased by 24% for cells treated with the antibiotic in concentration of 7.5 mM (EC_{50}), in comparison to the control cells (Fig. 4).

ROS are products of normal cells metabolism or xenobiotic exposure, and on concentrations, ROS can be beneficial or harmful to cells and tissues. At physiological low concentrations, ROS function as redox messengers in intracellular signaling and regulation while their excess induce oxidative modification of cellular macromolecules, inhibit protein function and promote cell death (33). A protective effect against drug-induced toxicity was provided by broad spectrum ROS scavengers, such as low molecular weight thiol compounds, vitamin E and salicylate (34). Melanin is known to be a scavenger of free radicals and it has been suggested that it possesses superoxide dismutase activity (35). Moreover, this biopolymer acts as a biochemical dustbin, mopping up potentially toxic agents (16). Such properties may be important for protecting the pigment cells as well as surrounding tissues from the natural toxins, xenobiotics, oxygen and ROS (including free radicals) (36). The ability of melanin to bind gentamicin used in low concentrations may prevent the antibiotic-induced toxic effects. The observed changes in antioxidant enzymes activity in cells exposed to the lowest gentamicin concentration (0.075 mM) confirmed that the antioxidative response is sufficient to compensate the increase in ROS formation. Under

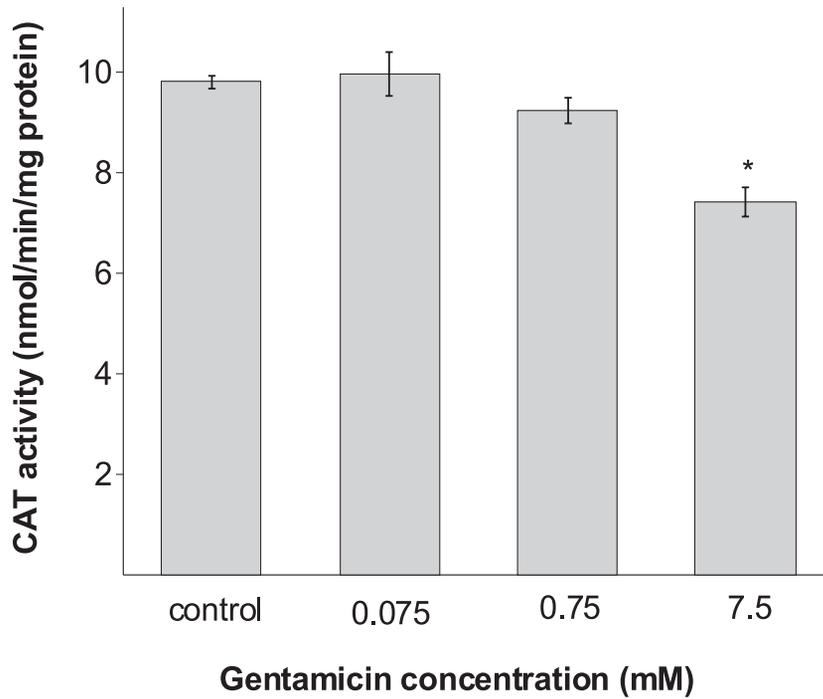


Figure 3. The catalase (CAT) activity in HEMn-DP cell after 24 h incubation with 0.075, 0.75 or 7.5 mM of gentamicin. Data are the mean \pm SEM of at least three independent experiments ($n = 3$) performed in triplicate. * $p < 0.05$ vs. the control samples

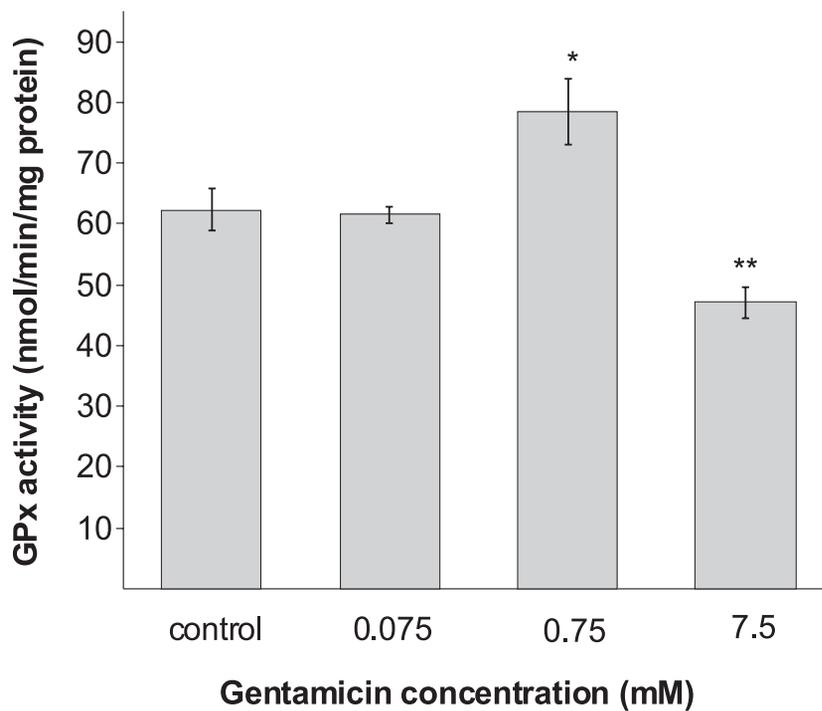


Figure 4. The glutathione peroxidase (GPx) activity in HEMn-DP cell after 24 h incubation with 0.075, 0.75 or 7.5 mM of gentamicin. Data are the mean \pm SEM of at least three independent experiments ($n = 3$) performed in triplicate. * $p < 0.05$ vs. the control samples; ** $p < 0.005$ vs. the control samples

certain conditions, when ROS production is increased more strongly and persistently, the antioxidative response may not be sufficient to provide the cellular redox homeostasis (33). The use of gentamicin in higher concentrations (0.75 and 7.5 mM) induced depletion of antioxidant defense system and therefore it could be further concluded that the prolongation of treatment with the high concentration of gentamicin may augment the toxic effect of aminoglycosides on pigmented tissues of the inner ear.

Previously, we documented that other aminoglycoside antibiotics, namely kanamycin (25) and streptomycin (27) caused depletion of antioxidant status of light pigmented normal human melanocytes (HEMa-LP). The observed changes in antioxidant enzymes activity were much more higher than in HEMn-DP cells under gentamicin treatment. The results of our prior studies (25, 27) and those described in this work reveal that the large amount of melanin present in dark pigmented melanocytes may prevent cells against the ROS induced toxic effects. In addition, this phenomenon could be confirmed by the lower gentamicin cytotoxicity ($EC_{50} = 7.5$ mM) in regard to kanamycin ($EC_{50} = 6.0$ mM) and streptomycin ($EC_{50} = 5.0$ mM), what may explain a protective role of melanin in the mechanisms of aminoglycosides ototoxic effects.

CONCLUSION

In summary, the present work provides the first *in vitro* study of the mechanisms involved in gentamicin-induced toxic effects on pigmented tissues using HEMn-DP melanocytes. Based on the obtained results concerning the effect of gentamicin on antioxidant enzymes activity in melanocytes, the potential role of melanin and melanocytes in the mechanisms of aminoglycosides toxic effects directed to pigmented tissues was determined, especially during high-dose and/or long-term therapy.

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