Mechanisms of cisplatin ototoxicity: theoretical review

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Abstract
Introduction: Cisplatin is an effective chemotherapeutic agent commonly used in the treatment of malignant tumours, but ototoxicity is a significant side effect.

Objectives: To discuss the mechanisms of cisplatin ototoxicity and subsequent cell death, and to present the results of experimental studies.

Material and methods: We conducted a systematic search for data published in national and international journals and books, using the Medline, SciELO, Bireme, LILACS and PubMed databases.

Results: The nicotinamide adenine dinucleotide phosphate oxidase 3 isoform (also termed NOX3) seems to be the main source of reactive oxygen species in the cochlea. These reactive oxygen species react with other molecules and trigger processes such as lipid peroxidation of the plasma membrane and increases in expression of the transient vanilloid receptor potential 1 ion channel.

Conclusion: Cisplatin ototoxicity proceeds via the formation of reactive oxygen species in cochlear tissue, with apoptotic cell death as a consequence.

Key words: Toxicity; Cisplatin; Cochlea; Hearing Loss; Free Radicals

Introduction
Cisplatin (cis-diaminedichloroplatinum II) is a highly effective chemotherapeutic agent commonly used in the treatment of several malignant tumours, especially those of the head and neck, in adults and children. Cisplatin acts in the tumour cell through mechanisms such as DNA damage and production of reactive oxygen species, which lead to cell death by apoptosis. Cell death can also occur via necrosis when the cell is exposed to high concentrations of cisplatin.1

The clinical administration of cisplatin is limited by its side effects, which include nephrotoxicity, ototoxicity, neurotoxicity, gastrointestinal tract toxicity and bone marrow toxicity (i.e. myelosuppression).3 Nephrotoxicity can be managed with hydration and administration of mannitol, diuretics or acetozolamide, and thiol compounds.3 Some other side effects can be mitigated with fractionated doses of medication.6 However, there appears to be no effective medical treatment for the prevention or control of ototoxicity, although the use of antioxidant therapy has been shown to be beneficial in animal models.8

Histopathological studies of cisplatin ototoxicity indicate that damage to the outer hair cells progresses from the base to the apex of the cochlea and from the third to the first row of these cells, after which damage progresses to the inner hair cells.9,10 Damage is not limited to the hair cells – the supporting cells, stria vascularis and spiral ganglion are also affected.3 However, Lee et al.11 have suggested that the stria vascularis of the cochlea is the region initially affected, leading to impaired uptake and secretion of potassium into the endolymph as well as impairment of the metabolic homeostasis of inner and outer hair cells, with resultant structural and functional damage.

The dose of cisplatin is an important determinant of the extent of ototoxic damage, a relationship first described in the 1980s and 1990s. When low doses were used, the stereocilia tip-link connections were damaged first, followed by disorganisation and fusion of stereocilia.12 High doses result in mitochondrial and endoplasmic reticulum damage, loss of stereocilia and of the hair cell itself, atrophy of the stria vascularis, collapse of Reissner’s membrane, and damage to supporting cells.13 Neurotoxicity occurs via a direct
action on neurons, or via a secondary mechanism whereby hair cell toxicity results in the loss of trophic factors produced by healthy cells and supplied to the neurons that innervate them.14

Besides the dose, other factors may affect the variability and severity of cisplatin ototoxicity, including patient age, renal function, interaction with other substances (e.g. antibiotics and diuretics), aminoglycosides, pre-existing hearing loss, and duration and form of cisplatin infusion.15

Clinically, cisplatin causes irreversible, bilateral, sensorineural hearing loss associated with tinnitus, which affects primarily the high frequencies and subsequently the low ones.16 High frequency hearing loss has been reported to affect between 50 per cent17 and 100 per cent18 of patients. Children are more susceptible than adults.19

Given this framework, the current study aimed to discuss the mechanisms of cisplatin ototoxicity and consequent cell death, as well as to present the results of experimental research.

Method
Systematic collection of data from national (i.e. Brazilian) and international journals was undertaken, using the Medline, SciELO, Bireme, LILACS and PubMed databases. Sixty-six relevant papers were selected, published from the 1980s to the present day, comprising 54 international and 12 national papers. We also obtained information from one internationally published and two Brazilian books.

Literature review
Free radicals due to cisplatin in the cochlea
There are several different mechanisms by which cisplatin damages the auditory system and triggers the apoptotic cell death pathway. One of these relates to the following sequence: covalent binding of cisplatin to guanine bases in DNA; formation of inter- and intra-strand chain crosslinking; induced p53; cell cycle arrest; and apoptosis. A second mechanism, currently widely discussed, refers to the generation of free radicals, specifically reactive oxygen species, which can increase lipid peroxidation, alter enzyme and structural proteins, and cause apoptotic cell death.16

Free radicals are atoms or molecules that contain an unusual number of electrons in their outer electron layer because they have undergone the process of oxidation (i.e. electron loss); thus, free radicals are products of oxidation.20 The non-pairing of the electrons of the outer layer confers high reactivity to these atoms or molecules.21

Oxidation is a fundamental part of human metabolism.22 Reactive oxygen species are formed by normal cellular metabolism (e.g. mitochondrial respiration), inflammatory processes, and in response to irradiation, chemotherapeutic agents and some antibiotics.23 Although oxygen radicals play an essential biological role, they are also potent oxidants, subtracting electrons from other molecules that interact with them and thereby converting those molecules into free radicals themselves, thus creating a cascade of damage which has been termed ‘oxidative stress’.24

In order to combat the reactive oxygen species formed by cellular respiration or in response to xenobiotics, as well as to keep the oxidative system balanced, cells use an endogenous antioxidant system. This system consists of enzymes, such as reduced glutathione, superoxide dismutase, catalase and glutathione peroxidase, which act before cell damage occurs and play a detoxification role. Ross and Moldeus have described a defence system which includes ascorbic acid, glutathione reductase and glutathione peroxidase, among others, and which acts to repair the damage caused by reactive oxygen species.25

Cochlear antioxidant systems include glutathione, glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase.7

Once generated, and if not controlled by antioxidant systems, reactive oxygen species can react with a variety of cellular components and cause damaging alterations, such as aldehydic lipid peroxidation of membranes, oxidative modifications of proteins, and DNA lesions.26

In the cochlea, cisplatin accumulates in the tissue, integrates with cellular DNA and causes dysfunction in protein synthesis, including antioxidant enzymes. Since the cochlea is in an isolated anatomical position, and effectively functions almost as a closed system, it is unable to expel the toxin at the same rate at which it is accumulated. Thus, an overload of reactive oxygen species develops, associated with an impaired antioxidant system.27 This state results in increased lipid peroxidation, triggering events that initiate the apoptosis of hair cells, supporting cells, stria vascularis and the auditory nerve.21

Degeneration of the stria vascularis is one of the first events in the development of cisplatin-induced hearing loss. Reactive oxygen species in the stria vascularis cause mitochondrial membrane permeabilisation and apoptosis of marginal cells.11

Apoptotic cell death (which is beneficial in cancer cells) is the basis for understanding cisplatin toxicity in healthy tissues such as the cochlea.29

The nicotinamide adenine dinucleotide phosphate oxidase 3 isoform (also termed NOX3) is now thought to play a central role in oxidation within the cochlea. This isoform is exclusively found in the inner ear and appears to contribute significantly to the generation of reactive oxygen species within the cochlea.30,31

In organotypic cultures, when activated by cisplatin, the nicotinamide adenine dinucleotide phosphate oxidase 3 isoform produces superoxide radical (O$_2^-$).31,32 The excess of reactive oxygen species in the cochlear tissue can react with nitric oxide to generate peroxynitrite, which can inactivate proteins.11

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Furthermore, superoxide radical may form hydroxyl radical (OH•) in its free form, and this ion may react with unsaturated fatty acids in the lipid bilayer of the cell membrane to generate aldehyde 4-hydroxynonenal, which is highly toxic and may lead to cell death. The increase in aldehyde 4-hydroxynonenal concentration is associated with an increased calcium influx to the outer hair cells, leading to apoptosis.33 The superoxide anion may also inactivate antioxidant enzymes34 and cause migration of the pro-apoptotic Bax protein to the cytosol, resulting in the release of cytochrome c from the damaged mitochondria and the consequent activation of caspases 9 and 3.7

In addition to the above-mentioned plasma membrane lipid peroxidation and effects on cochlear tissue, reactive oxygen species may also activate and increase the expression of the transient vanilloid receptor potential 1 ion channel (also termed receptor transient potential V1).30 This is a member of the transient receptor potential family of ion channel proteins, and is expressed primarily in small diameter neurons35 and non-neuronal tissues (e.g. the organ of Corti).36,37 Increased expression of transient vanilloid receptor potential 1 contributes to cell death by increasing the influx of calcium into the cell, with consequent calcium overload, and by activation of caspases.16,30

Apoptotic cell death due to reactive oxygen species in the cochlea

Apoptosis of cochlear cells may be precipitated by the complex interaction between cisplatin and the DNA of the damaged cell, preventing normal cell cycle progression.38 In addition, drug-induced oxidative stress triggers a cascade of intracellular reactions that culminates in apoptosis.39

Apoptosis is a cell death pathway induced by a highly regulated cellular programme4 involving programmed cell death and ‘suicide’ cells.40 It can be triggered by several physiological situations, such as the destruction of cells during embryogenesis,41 or due to factors such as chemotherapeutic agents, ionising radiation, DNA damage, heat shock, growth factor deprivation, nutrient deficiency, binding of molecules to membrane receptors, and increased levels of reactive oxygen species.41

The morphological and biochemical characteristics of apoptosis involve nuclear fragmentation, chromatin condensation, cell shrinkage, ‘bubbling’ of the cellular surface with maintenance of membrane integrity, apoptotic body formation and proteolysis (i.e. protein degradation by enzymes). Cell debris are phagocytosed by neighbouring cells or macrophages.42

For some years, the specific intracellular proteases of the caspase family have been investigated as crucial effectors of apoptosis.43

The caspases (which derive their name from cysteine aspartic acid proteases) are based on cysteine proteases able to cleave other proteins after an aspartic acid residue,44 and are synthesised as inactive precursors known as zymogens (pro-caspase).41 The caspase family can be divided functionally into initiator caspases and effector caspases, depending on the order in which they are activated during apoptosis. The initiator caspases include types 8 and 9, while the effector caspases include types 3, 6 and 7, among others.24

The pro-caspases are activated by proteolytic cleavage of caspases when the cell receives a signal for cell death.45 Once activated, they operate in a cascade fashion, that is, the initiator caspases activate the effector caspases which in turn effect the fragmentation of cellular DNA.46

The apoptotic process can be triggered by two pathways, one extrinsic (cytoplasmic) and the other intrinsic (mitochondrial), both of which converge to activate effector caspases.47 Furthermore, these routes can be classified according to the type of pro-caspase that is activated. In the extrinsic pathway, activation of pro-caspase 8 (an initiator caspase) results from signalling of a cell surface death receptor, such as Fas or tumour necrosis factor receptor 1. In the intrinsic pathway, the activation of pro-caspase 9 (an initiator caspase) is mainly dependent on the mitochondrial signalling pathway regulated by members of the Bcl-2 (B-cell lymphoma 2) family.48 The Bcl-2 family is composed of 25 anti- and pro-apoptotic members.49

The activation of the pro-apoptotic members of the Bcl-2 family, such as Bax protein, may trigger a sequence of events that lead to changes in mitochondrial permeability and the release of cytochrome c (a caspase activator protein) into the cytoplasm, which binds to another protein (protease activating factor in apoptosis 1, also termed ‘Apaf-1’) to form a protein complex (apoptosome) that activates pro-caspase 9.50

Once activated, caspases 8 and 9 both participate in a cascade of events leading to the activation of caspase 3, which cleaves various substrates and results in DNA fragmentation and the morphological characteristics of cell apoptosis.51 In contrast, the Bcl-2 protein, a member of the anti-apoptotic Bcl-2 family, promotes cell survival by preventing the escape of cytochrome c into the cytoplasm, possibly by forming heterodimers with pro-apoptotic molecules such as Bax protein.52

Decreased transcription of the anti-apoptotic protein Bcl-2 and increased transcription of the pro-apoptotic Bax protein are mediated by the p53 gene. This is a tumour suppressor gene that accumulates in the cell when DNA is damaged, causing suspension of the cell cycle at the G1 phase and facilitating DNA repair. However, if the repair fails, p53 triggers apoptosis by stimulating the transcription of pro-apoptotic proteins and decreasing the transcription of anti-apoptotic proteins.24

The mitochondrial dysfunction that triggers the intrinsic apoptosis pathway can be caused by intracellular stress signals resulting from DNA damage, toxins or oxidative stress, among others.53
Devarajan et al.\textsuperscript{38} have confirmed the involvement of both extrinsic pathway (death receptor) and intrinsic pathway (mitochondrial) apoptosis induced by cisplatin in an \textit{in vitro} model of auditory cells.

\textbf{Results of experimental research}

Several studies on the auditory effects of cisplatin have been conducted. Important findings are summarised below, to provide an overview and to help guide subsequent studies.

In 1995, Ravi, Somani and Rybak\textsuperscript{39} induced ototoxicity in rats by administering different doses of cisplatin. These authors found that the cisplatin ototoxicity was associated with decreased activity of glutathione peroxidase and glutathione reductase, compared with controls. They also found increased activity of superoxide dismutase and catalase three days after cisplatin administration, suggesting an increased concentration of reactive oxygen species within the cochlea and an impaired cochlear antioxidant system. There was also an accumulation of malondialdehyde, an indicator of lipid peroxidation.

Alam et al.\textsuperscript{54} administered cisplatin (4 mg/kg/day) for 5 consecutive days and subsequently identified apoptotic cells in all cochlear structures, including the inner and outer hair cells, supporting cells, spiral ganglion, stria vascularis, and spiral ligament. An increase in the expression of Bax protein and a decrease in Bcl-2 suggested the importance of the Bcl-2 protein family in the control of cisplatin-related apoptosis. In addition, Liu et al.\textsuperscript{55} and Cheng et al.\textsuperscript{56} studied organ of Corti cell cultures from 3-day-old rats and demonstrated the role of apoptosis as a mechanism of cisplatin-induced cell damage.

The extent and degree of histological and functional auditory system damage appear to be related to the cisplatin dose administered. Different methodologies have been studied, in guinea pig models, in order to investigate this relationship. Applying a dosage of 1 mg/kg/day for 4, 8, 12 and 16 days, Schweitzer\textsuperscript{1} suggested that a cumulative dose of 16 mg/kg was required to cause maximal cell loss (78 per cent in this study). Cardinale et al.\textsuperscript{57} reported maximal (i.e. 65 per cent) loss of the outer hair cells of the basal turn of the cochlea following a dosage of 2 mg/kg/day for eight days (also representing a cumulative dose of 16 mg/kg).

In order to establish the optimal cisplatin dose, and dose administration, required to achieve significant cell damage, Ilha et al.\textsuperscript{6} studied 48 female albino guinea pigs divided into four groups (each containing nine animals in the final analysis) receiving four different intraperitoneal cisplatin regimes: (1) a single dose of 7.5 mg/kg/day; (2) two doses of 7.5 mg/kg/day for the first and fifth day; (3) three doses of 7.5 mg/kg/day for the first, fifth and sixth day; or (4) doses of 2.5 mg/kg/day for the first, second, third, fourth, fifth and sixth day. Animals underwent distortion product otoacoustic emission (DPOAE) testing during cisplatin treatment. Following sacrifice, the animals’ cochleae were analysed by scanning electron microscopy. The authors found lesions in 70 per cent of the outer hair cells in the first and second turns in animals receiving 7.5 mg/kg/day cisplatin on the first, fifth and sixth days. These findings were confirmed by DPOAE results. Despite these substantially auditory effects, other clinical effects were such that animals were still able to be kept for periods of up to 21 days. This dosing regimen was used in further studies, together with otoprotectors.\textsuperscript{58}

Rybak et al.\textsuperscript{59} studied rats treated with a single dose of 16 mg/kg cisplatin, and found significant changes in brainstem evoked auditory potential threshold and decreased intracellular glutathione levels (to 53 per cent in a control group). Decreased levels of antioxidant enzymes were also found, i.e. superoxide dismutase (to 52 per cent of the control level), glutathione reductase (to 50 per cent of the control level), glutathione peroxidase (to 70 per cent of the control level), and catalase (to 70 per cent of the control level) and glutathione peroxidase (to 50 per cent of the control level). Membrane lipid peroxidation was also observed, as indicated by a malondialdehyde level increase to 165 per cent compared with the control group level.

Freitas et al.\textsuperscript{60} investigated the role of apoptosis in cisplatin-induced cochlear injury using a male Wistar rat model. The animals were divided into eight groups distinguished by differing cisplatin dose, administration mode, and assessment type and timing (DPOAE and brainstem evoked auditory potentials were used). The most relevant of these test results showed that, in mice receiving 8 mg/kg/day of cisplatin for 3 consecutive days (a cumulative dose of 24 mg/kg), there was a significant decrease in DPOAE amplitudes on the third day, but that animals receiving a single, 16 mg/kg dose showed no such result. A significant increase in electrophysiological thresholds was observed on the third and fourth day after receipt of either a single cisplatin dose of 16 mg/kg or a cumulative dose of 24 mg/kg. This study also performed immunohistochemical analysis of tissue using the membranous labyrinth terminal deoxyribonucleotidyl transferase mediated deoxyuridine triphosphate digoxigenin nick end labelling assay (also termed TUNEL assay), which showed significantly greater staining intensity in the cochleae of animals treated with a single cisplatin dose of 16 mg/kg. Apoptosis immunostaining was seen in the inner and outer hair cells, supporting cells, stria vascularis, spiral ligament, spiral limbus, and spiral ganglion.

Cisplatin-induced cochlear cell damage shows two peculiarities: firstly, the outer hair cells of the cochlear base are inherently more susceptible to free radical damage than the outer hair cells of the cochlear apex; and secondly, supporting cells have considerably greater survival capacity than sensory cells. Sha et al.\textsuperscript{61} reported that glutathione levels were higher in the apical outer hair cells compared with the basal cells, which suggests a differential susceptibility to
damage via varying sensitivity to the action of reactive oxygen species. However, Usami et al. reported that the protective mechanisms provided by endogenous cellular antioxidants such as glutathione and enzymes such as superoxide dismutase and glutathione peroxidase provide a primary defence against free radicals. This protective mechanism may be distributed differently within various cells of the cochlea.

Given the irreversible nature of cisplatin-induced hearing loss, other studies have investigated the action of potentially otoprotective substances coadministered with cisplatin, with some promising positive results. Such potentially otoprotective substances include fosfomycin, sodium thiosulfate, amifostine, Ginkgo biloba extract, vitamin E and N-acetylcysteine, among others.

**Conclusion**

The presence of cisplatin triggers a series of events in the cochlea which are harmful to cell survival. There is evidence that this process begins with activation of the nicotinamide adenine dinucleotide phosphate oxidase 3 isoform, which is considered the main source of reactive oxygen species within the cochlea, with consequent impaired synthesis of the antioxidant enzymes which normally play a fundamental role in cellular oxidative balance. Thus, cochlear tissue is placed under oxidative stress, with the production of reactive oxygen species working in opposition to the antioxidant system.

Reactive oxygen species cause damage via plasma membrane lipid peroxidation and alteration of proteins and DNA. When these lesions are not repaired by the cellular defence system, the cell crosses the threshold of reversible injury and recognises that repair has failed; thereafter, activation of the apoptosis pathway programmes the cell to die.

The biochemical process of apoptosis proceeds via two main pathways: extrinsic (also termed cytoplasmic) and intrinsic (also termed mitochondrial). Both pathways converge to produce caspase 3 activation, DNA fragmentation and formation of apoptotic bodies which are then phagocytosed.

This paper aims to discuss some of the important aspects of the cisplatin ototoxicity mechanism. However, there is still much to be clarified, and the continuation of experimental research is essential for this purpose.

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