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Assessing cisplatin-induced ototoxicity and otoprotection in whole organ culture of the mouse inner ear in simulated microgravity

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ABSTRACT

Cisplatin is a widely used anti-cancer drug. Ototoxicity is a major dose-limiting side-effect. A reproducible mammalian *in-vitro* model of cisplatin ototoxicity is required to screen and validate otoprotective drug candidates. We utilized a whole organ culture system of the postnatal mouse inner ear in a rotating wall vessel bioreactor under "simulated microgravity" culture conditions. As previously described this system allows whole organ culture of the inner ear and quantitative assessment of ototoxic effects of aminoglycoside induced hair cell loss. Here we demonstrate that this model is also applicable to the assessment of cisplatin induced ototoxicity. In this model cisplatin induced hair cell loss was dose and time dependent. Increasing exposure time of cisplatin led to decreasing EC_{50} concentrations. Outer hair cells were more susceptible than inner hair cells, and hair cells in the cochlear base were more susceptible than hair cells in the cochlear apex. Initial cisplatin dose determined the final extent of hair cell loss irrespective if the drug was withdrawn or continued. Dose dependant otoprotection was demonstrated by co-administration of the antioxidant agent N-acetyl L-cysteine. The results support the use of this inner ear organ culture system as an *in vitro* assay and validation platform for inner ear toxicology and the search for otoprotective compounds.

1. Introduction

In a recent survey of the International Agency for Research on Cancer 3.2 million cases of cancer were newly diagnosed in the European countries (Ferlay et al., 2010). In the treatment of cancer, cisplatin [cis-Diammin dichloroplatinum (II)] has remained an effective and widely used anti-cancer drug since it was first introduced to the clinic in the early 1970s. Applications include a widespread range of cancers in children and adults. Adverse side-effects of cisplatin include nephrotoxicity, bone marrow toxicity, gastrointestinal toxicity, liver toxicity and neurotoxicity (Ekborn et al., 2003). Beside these, one of the major dose-limiting side effects is ototoxicity, often resulting in irreversible sensorineural hearing loss. For example, 20% of testicular cancer patients treated at standard dose experience persistent ototoxicity but it may affect more than 50% of patients receiving cumulative high doses of cisplatin > 400 mg (Bokemeyer et al., 1998). Up to 60% of paediatric cancer patients treated with cisplatin develop permanent bilateral hearing loss (Knight et al., 2005, 2007; Brock et al., 2012). In children, young age, male gender and increasing cumulative dose appear as critical risk factors for cisplatin induced ototoxicity (Yancey et al., 2012).

Cisplatin induced ototoxicity in the mammalian inner ear has been described and modelled in numerous animal studies (reviewed in: Rybak et al., 2007, 2009, 2012; Rybak and Ramkumar, 2007; Rybak and Whitworth, 2005; Schacht et al., 2012). In the cochlea cisplatin causes degeneration of three different types of cells; the hair cells in the organ of Corti (Anniko and Sobin, 1986; Fleischman et al., 1975; Zheng and Gao, 1996) the epithelial cells of

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the stria vascularis (Meech et al., 1998; Ravi et al., 1995) and neurons of the spiral ganglion (van Ruijven et al., 2005; Zheng and Gao, 1996; Zheng et al., 1995). A more profound toxic effect of cisplatin on SGN than on hair cells was found in experiments with cochlear explant cultures (Zheng and Gao, 1996). It is generally accepted that cisplatin ototoxicity in hair cells is mediated by reactive oxygen species (ROS) (Clerici et al., 1996; Dehne et al., 2001; Kim et al., 2010; Rybak, 2007; Rybak et al., 2009; Rybak and Ramkumar, 2007). Transcription factor NF-κB and high-mobility group (HMG) protein (Rybak and Ramkumar, 2007), transcription factor STAT1 (STAT; signal transducers and activators of transcription) (Kaur et al., 2011; Schmitt et al., 2009) and Nox3 (Rybak et al., 2012) are suggested as further death pathways. Currently, large scale drug screening for otoprotective agents in *in vivo* animal models is impeded by the mortality related to the systemic toxicity of cisplatin and the inter-individual variability of the observed ototoxic effects particularly in mice (Parham, 2011). Furthermore, the number of animals necessary to fully evaluate the number of potential ototoxic and otoprotective agents is prohibitive. Towards the development of an *in vivo* model a quantitative assay employing the non-mammalian model of the lateral line of zebrafish larvae has successfully been introduced to facilitate drug screening for ototoxic and otoprotective agents (Chiu et al., 2008; Hirose et al., 2011a; Ou et al., 2007; Ton and Parng, 2005). Hit compounds identified in the zebrafish screening system need to be validated in mammalian *in vitro* models.

Mammalian *in vitro* models to study cisplatin ototoxicity have utilized inner ear cell lines, tissue culture and to a certain extent whole organ culture. Immortalized cell lines have been generated from the auditory sensory epithelia of the H-2Kb-tsA58 transgenic mouse (Jat et al., 1991) at embryonic (Rivolta et al., 1998), postnatal (Kalinec et al., 2003) and functionally mature, adult-like (Kalinec et al., 1999) developmental stages. These cell lines have been employed for several purposes including the screening for toxic and protective agents (Kalinec et al., 2003; Rivolta and Holley, 2002). Tissue culture of isolated tissue fragments dissected from the postnatal inner ear has been used extensively for the study of cisplatin ototoxicity (Ding et al., 2011; Liu et al., 1998; Park et al., 2009; Previati et al., 2007; Yarin et al., 2005; Zhang et al., 2003). However, tissue culture models require microdissection of the auditory sensory epithelium which is technically demanding and time-consuming and puts a limit to its application as a validation tool for the increasing number of potential ototoxic and otoprotective agents that arise from the increasing number of screening efforts using inner ear derived cell lines or the zebrafish lateral line. Finally, organ culture models used for the study of cisplatin ototoxicity have been limited to developmental stages of the late embryonic inner ear (Anniko and Sobin, 1986).

In the present study we utilized our recently developed whole organ culture model of the postnatal inner ear in a rotating bioreactor culture system under "simulated microgravity" conditions (Arnold et al., 2010; Hahn et al., 2008). This system allows maintaining the entire mouse inner ear organ isolated at postnatal day 7 for up to seven days *in vitro* culture (Hahn et al., 2008). Here we demonstrate that in this model ototoxicity can be quantitatively assessed for cisplatin and also serve as a validation platform for otoprotective compounds such as the antioxidant agent N-acetyl L-cysteine (L-NAC). L-NAC is in clinical use for many years (review: Samuni et al., 2013). It is frequently prescribed as a mucolytic and antioxidative agent, due to its rapid reactions with oxygen and thiyl radicals. L-NAC has recently (Riga et al., 2013; Yoo et al., 2013) been tested clinically in patients receiving cisplatin treatment. It was shown that L-NAC was at least a promising drug to prevent cisplatin-induced ototoxicity.

2. Material and methods

2.1. Animals

Mice used this study were obtained from an in house breeding colony of either a 129SV (cisplatin ototoxicity) or NMRI (otoprotection) genetic background. Both mice strains gave similar results regarding cisplatin ototoxicity. One hundred fifty six animals were used in this study. Animal use for organ explantation was approved by the Committee for Animal Experiments of the Regional Council (Regierungspräsidium) of Tübingen (dated 19 October 2010 and 06 November 2012).

2.2. Organ culture system setup

Details of the methods were described previously (Arnold et al., 2010; Hahn et al., 2008). Briefly, inner ear organ explants were obtained from mice aged postnatal day 7 and 5–6.5 g bodyweight. Whole organ inner ear explants were cultured in 55 ml High Aspect Ratio Vessel (HARV; Synthecon Inc., Houston, TX, USA) culture vessels mounted on a Rotary Cell Culture System (RCCS™-4; Synthecon Inc., Houston, TX, USA). All dissections were carried out under sterile conditions in a laminar flow cell culture hood (HeraSafe KS12, Heraeus Instruments GmbH, Hanau, Germany). Mice pups at postnatal day 7 were decapitated. The complete inner ear bony labyrinth capsules were dissected from the skull base in ice-cold Hank's balanced saline solution (HBSS). After completion of the gross dissection, a micro-dissection opening the perilymphatic fluid spaces was carried out in order to provide access of the culture medium to the inner ear sensory epithelia. The bioreactor was placed in a 37 °C, humidified 5% CO_2/95% air incubator (HeraCell CO2 Incubator, Heraeus Instruments GmbH, Hanau, Germany). Each incubator was installed with one RCCS™-4 allowing the culture of up to four culture vessels in parallel in one incubator. Freshly dissected inner ear organ explants were inserted into the HARV culture vessels and suspended in cell culture medium. The HARV vessel was running at a rotation speed of 30 rounds/min (RPM). The culture medium was Neurobasal® Medium (Life Technologies™, Darmstadt, Germany) supplemented with 1× B27® supplement (Life Technologies™, Gibco®, Darmstadt, Germany) 5 mM glutamine (Life Technologies™, Invitrogen, Inc., Darmstadt, Germany), 10 mM Hepes (Life Technologies™, Invitrogen, Inc., Darmstadt, Germany), and 100 U of penicillin (Sigma, St. Louis, MO, USA).

2.3. Application of cisplatin

Cisplatin (MW: 300.05 g/mol, Sigma–Aldrich, St. Louis, MO, USA; thus 1 μg/ml = 3.3 μM) was applied to the culture medium at different concentrations (up to 30 μg/ml, corresponding to approx. 99 μM) for 24, 48 or 96 h. In detail, concentrations used for 24 h were 1, 2.5, 5, 10, 15 and 30 μg/ml (corresponding molar concentrations: 3.3, 8.25, 16.5, 33, 49.5 and 99 μM), for 48 h 0.1, 0.3, 0.5, 1, 1.5, 1.75, 2, 2.5, 3 and 5 μg/ml (corresponding molar concentrations: 0.33, 0.99, 1.65, 3.3, 4.95, 5.77, 6.6, 8.25, 9.9 and 16.5 μM) and for 96 h 0.1, 0.3, 0.5, 1 and 2 μg/ml (corresponding molar concentrations: 0.33, 0.99, 1.65, 3.3, and 6.6 μM).

2.4. Application of L-NAC

The otoprotective compound L-NAC (N-acetyl-L-cysteine; Sigma–Aldrich, St. Louis, MO, USA) (Feghali et al., 2001; Ton and Parng, 2005) was applied at a concentration of 0.1, 0.3, 1 and 3 mM. For this series of experiments separate controls were made without