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Agmatine alleviates cisplatin-induced ototoxicity by activating PI3K/AKT signaling pathway

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Running title: Agmatine alleviates cisplatin-induced ototoxicity

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Abstract
Cisplatin-induced ototoxicity can be partially attributed to excessive reactive oxygen species (ROS) production, and agmatine is well-known for the activation of the phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (AKT) pathway to inhibit ROS production. Whether agmatine could be utilized to alleviate cisplatin-induced ototoxicity is investigated. Cisplatin exposed House Ear Institute-Organ of Corti 1 (HEI-OC1) cells and cochlear explants showed increased ROS production detected by 2′-7′dichlorofluorescin diacetate (DCFH-DA) staining and decreased cell viability detected by Cell Counting Kit-8 (CCK-8) or Myosin 7a staining, which could be reversed by the agmatine pre-treatment. Cisplatin intraperitoneally injected C57BL/6 mice demonstrated damaged auditory function as indicated by distortion products otoacoustic emissions (DPOAE) and auditory brainstem response (ABR) assays, and trans-tympanically administrated agmatine in the left ears could partly prevent the auditory function loss. Mechanistically, down-regulated B-cell lymphoma 2 (Bcl-2) expression, up-regulated Bcl2 Associated x (Bax) expression, and diminished p-PI3K and p-AKT expression were detected in cisplatin-exposed HEI-OC1 cells and cochlear explants, which could be prevented by the pre-treatment with agmatine. Our investigation demonstrates that agmatine pre-treatment could alleviate cisplatin-induced ototoxicity with the activation of PI3K/AKT signaling pathway.

Keywords: Agmatine; cisplatin-induced ototoxicity; PI3K/AKT
Significance Statement

The study demonstrated that agmatine pre-treatment could activate PI3K/AKT signaling pathway to alleviate cisplatin-induced ototoxicity.
Cisplatin was approved in 1978 by the US Food and Drug Administration (FDA) to treat ovarian cancer and metastatic testicular patients (Farrell 2015). Although serious side effects such as bone marrow depression, nephrotoxicity, and ototoxicity can occur, cisplatin remains the most widely utilized and available chemotherapeutic drug to treat solid malignant tumors (Glynne-Jones and Hoskin 2007, Chang and Chinosornvatana 2010, Johnstone, Suntharalingam et al. 2016, Rottenberg, Disler et al. 2021). The incidence of cisplatin-induced ototoxicity can range from twenty percent to seventy percent, which may manifest with progressive, irreversible, and bilateral hearing loss (Tang, Wang et al. 2021). Young children are more inclined to cisplatin-induced ototoxicity with delayed speech development and psychosocial and cognitive development (Knight, Kraemer et al. 2005, Rybak, Mukherjea et al. 2019).

It is generally believed that cisplatin-induced ototoxicity may be attributed to the excessive reactive oxygen species (ROS) production by the cochlea (Yu, Gu et al. 2020), and endoplasmic reticulum stress is a target for treatment of hearing loss (Wang and Xu 2020). Multiple promising strategies have been performed to alleviate, treat, and prevent cisplatin-induced ototoxicity, and none of these strategies has been confirmed or recommended by the FDA (Gentilin, Simoni et al. 2019, Mukherjea, Dhukhwa et al. 2020).

Among the multiple signaling pathways contributing to the survival and differentiation of hair cells, phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT) pathway is well investigated (Liu, Wei et al. 2021), which might play an essential role in inner ear hair cells survival to resistance against harmful stimuli (He, Zheng et al. 2021). It is worth
noting that, in neonatal cochlear spiral ganglion explants, PI3K/AKT signaling mediates brain-derived neurotrophic factor-induced neurite formation (Mullen, Pak et al. 2012). In the noise-induced cochlea injury, PI3K/AKT pathway activation induced by deferoxamine may promote mesenchymal stem cell homing (Peyvandi, Abbaszadeh et al. 2018).

Agmatine is formed by L-arginine decarboxylation and hydrolyzation to putrescine, which can bind to N-methyl-D-aspartate (NMDA) receptors and α2-adrenergic receptors to function as novel neurotransmitters and neuromodulators (Regunathan 2006, Piletz, Aricioglu et al. 2013). Substantial preclinical and initial clinical evidence has indicated the possibility to treat opioid addiction, mood disorders, neurotrauma, neurodegenerative diseases, and cognitive disorders (Xu, Gao et al. 2018, Akasaka and Fujiwara 2020).

This investigation utilizes agmatine to treat cisplatin exposed HEI-OC1 cells, cochlear explants, or cisplatin affected mice and finds that agmatine alleviates hearing loss with reduced ROS production and cell loss and up-regulated PI3K/AKT signal pathway. Therefore, as an adjuvant drug, agmatine has the potential value in reducing ototoxicity caused by cisplatin chemotherapy.

Methods & materials

Cell viability

HEI-OC1 cells (5, 000/well) were seeded in 96-well plates in three replicates, and relevant agmatine (10, 50, 100, 200 μM) and or cisplatin (5, 10, 30, 50 μM) were incubated for indicated hours. CCK-8 (Dojindo Laboratories) was added to each well with the final concentration of 10% for 4 hours. The optical density values were...
measured at 450 nm with a Bio-Rad plate reader.

**Cisplatin exposed HEI-OC1 cells culture**

HEI-OC1 cell line was pre-treated with 100 μM agmatine for 2 hours and then cotreated with 30 μM cisplatin for 24 hours in appropriate conditions (33°C, 5% CO₂, high-glucose DMEM medium, 5% fetal bovine serum, Gibco).

**ROS detection**

2’-7’dichlorofluorescin diacetate (DCFH-DA) working solution (10 μM, Beyotime, S0033) was added into six-well plates and incubated the plates at 37°C for 30 minutes. After the incubation, the fluorescence was observed with the LEXT OLS5100 laser scanning confocal microscope.

**Cisplatin exposed cochlear explants**

Cochleae from C57BL/6 mice (three days postnatal) were dissected out and seeded intact on Cell-Tak (BD Biosciences, Franklin Lakes, NJ, USA) coated glass coverslips, which were further incubated with DMEM/F12 medium supplemented with 1× N2/B27 as recommended by the manufacturer (Invitrogen, Waltham, MA USA) at 37°C with 5% CO₂. Agmatine (100 μM) was used to pre-treat cochlear explants for 2 hours, and then cisplatin (30 μM) was added to induce the ototoxicity for 24 hours.

**Western blot**

HEI-OC1 or cochlea explant lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene fluoride membranes, which was further blocked with 5% nonfat dry milk and incubated with primary antibodies against GAPDH, Bax, Bcl2, p-PI3K, p-AKT, PI3K, and AKT (Santa Cruz). Peroxidase-conjugated secondary antibody (Sigma-Aldrich, 1:1000 dilution, 2 hours, at
room temperature) was added, and an ECL system (Sigma-Aldrich) was utilized to obtain the signal. The intensity of protein bands was quantified with Image J software. GAPDH was utilized as the loading control to normalize the relative expression.

Cisplatin exposed mice

C57BL/6 male mice (four-week-old) purchased from Peking Vital River Laboratory Animal Ltd. (Beijing, China) were maintained. Agmatine (10 μM, 5 μL) was trans-tympanically injected into the left ears, while the same volume phosphate-buffered saline (PBS) was injected into the contralateral ears. Then cisplatin (30 mg/kg) was i.p. administered two hours later. Seven days post cisplatin administration, the auditory brainstem responses (ABR) and distortion product otoacoustic emission (DPOAE) measurements were done. All the procedure was approved by the Ethics Committee of the Second Hospital of Hebei Medical University.

ABR test

ABR assessment was performed as previously reported (McLean, Clamp et al. 2021). Briefly, anesthetized mice (25 mg/kg xylazine sodium and 100 mg/kg ketamine, i.p.) were kept warm during the ABR recordings process (highest intensity of acoustic stimuli, 90 dB SPL; decrements, 5 dB SPL) at 38°C on the thermostatic heating pad. The hearing threshold at five frequencies (4, 8, 16, 24, and 32 kHz) was detected with TDT System III apparatus (Tucker Davies Technologies).

DPOAE test

DPOAE was performed as previously reported with a TDT-RZ6 system (Tucker-Davis Technologies) (Li, Zhou et al. 2021). Two sine wave tones with different frequencies but equal intensities (F2=1.2F1, F2 ranging from 4 to 40 kHz) were utilized with 1 s duration.
to elicit DPOAE. Twenty adjacent frequency bins around the distortion product frequency were averaged as the surrounding noise floor. DPOAE threshold was determined when the signal was over 5 dB sound pressure level and over two standard deviations above the surrounding noise floor.

**Immunofluorescence**

Intact cochleae were separated from the temporal bone, which were embedded in Optimal Cutting Temperature O.C.T medium (Richard-Allan Scientific, Kalamazoo, MI), snap frozen in liquid nitrogen, and stored at −80 °C until use. Five-micrometer sections were cut by a cryostat (Microm HM525, Walldorf, Germany). After being fixed with 4% paraformaldehyde for 10 min, the sections were blocked with 10% normal goat serum and permeabilized with 0.3% Triton X-100 for 2 h at room temperature. The Fast ImmunoCytoChemistry Staining Kit (Protein Biotechnologies), anti-Myosin 7a antibody (Proteus Bioscience, 25–6790), and DAPI were utilized for hair cell detection.

**Statistical Analysis**

The difference between groups was assessed using one or two-way ANOVA analysis before corresponding post hoc tests. The significance level was set as $p$-value < 0.05. All statistical analyses were performed with GraphPad Prism (GraphPad Software).

**Results**

*Agmatine alleviates cisplatin-induced ototoxicity in HEI-OC1 cells*

To optimize the dose of cisplatin, different concentrations of cisplatin (0, 5, 10, 30, or 50 μM) were utilized to treat HEI-OC1 cells for 24 hours, and the cell viability was analyzed by CCK-8 assay. Cisplatin (at a dose greater than 30 μM) can markedly reduce cell
viability (Figure 1a). While as to agmatine, only 200 μM agmatine could diminish the viability of HEI-OC1 cells (Figure 1b), which indicated that the dose under 200 μM was safe. To determine the protective effect of agmatine on cisplatin-induced ototoxicity, HEI-OC1 cells were pre-treated with different concentrations of agmatine for 2 hours and then cotreated with 30 μM cisplatin for 24 hours. A significant dose-dependent protective effect was observed, and 100 μM agmatine showed the maximal protective effect (Figure 1c). These results testified that agmatine could protect HEI-OC1 cells viability upon cisplatin exposure, and 100 μM agmatine and 30 μM cisplatin were chosen in the following experiment.

**Agmatine alleviates cisplatin-induced ROS in HEI-OC1 cells**

We examined ROS production with a mitochondria-specific ROS indicator, DCFH-DA, to determine whether agmatine could alleviate cellular oxidative stress. Cisplatin-induced up-regulated ROS production, while the pre-treatment with agmatine could diminish ROS induction (Figure 2a and 2b). It was worth noting that agmatine alone could not induce the production of ROS. All of these indicated the protection effect caused by agmatine.

**Agmatine alleviates cisplatin-induced cochleae cell apoptosis in vitro with up-regulated PI3K /AKT pathway**

In order to decipher the relevant pathway for apoptosis, Bcl-2 family proteins expression was detected with Western blot in cisplatin exposed cells. Elevated Bax (pro-apoptotic, Figure 3a and 3b) and decreased Bcl-2 (anti-apoptotic, Figure 3a and 3c) were observed, which could be reversed by the pre-treatment of agmatine. At the same time, the upstream PI3K/AKT signaling pathway molecules were detected. After cisplatin
treatment, down-regulated p-PI3K (Figure 3a and 3d) and p-AKT expression (Figure 3a and 3e) were detected. As expected, agmatine administration could up-regulate the PI3K/AKT pathway as indicated by the up-regulation of p-PI3K and p-AKT. These data demonstrated that agmatine had the ability to inhibit cisplatin-induced apoptosis with up-regulated PI3K/AKT signaling pathway in HEI-OC1 cells.

**Agmatine alleviates cisplatin-induced cochleae explants apoptosis**

Myosin 7a staining showed that cisplatin treatment could lead to the conspicuous loss of mature hair cells in the apex (data not shown), basal turns (data not shown), and the middle turn of cochlea. The most significant damage effect was observed on the middle turn of the cochlea, which could be alleviated by the pre-treatment of agmatine (Figure 4a and 4b). Accumulation of ROS may lead to the apoptosis of hair cells. No DCFH-DA-positive cells were detected in the untreated or the agmatine-treated cochlear explants (Figure 4c). While significantly increased DCFH-DA-positive cells were detected after cisplatin exposure, this increase was reversed by agmatine pre-treatment (Figure 4c and 4d).

**Agmatine stimulates PI3K/AKT signaling to inhibit the apoptosis in cochleae explant induced by cisplatin exposure**

Cochleae explant was further utilized to confirm the apoptosis induced by cisplatin exposure. Increased Bax expression (pro-apoptotic, Figure 5a and 5b) and decreased Bcl-2 expression (anti-apoptotic, Figure 5a and 5c) were observed in cisplatin exposed cochleae explant, which could be reversed by the agmatine pre-treatment. At the same time, down-regulated p-PI3K (Figure 5a and 5d) and p-AKT expression (Figure 5a and 5e) were detected after cisplatin treatment. As expected, agmatine administration could
up-regulate the PI3K/AKT pathway. All of these data confirmed that agmatine could stimulate PI3K/AKT signaling pathway to inhibit cisplatin-induced intrinsic apoptosis pathway in cochleae explant and HEI-OC1 cells.

**Agmatine prevents auditory function loss in cisplatin exposed mice**

ABR and DPOAE measurements were utilized to indicate the auditory function. Hearing thresholds were significantly elevated at all frequencies tested 14 days after cisplatin exposure, whereas pre-treatment with agmatine could diminish the thresholds (Figure 6a and 6b). All of these indicated that agmatine could partially prevent auditory function loss in cisplatin exposed mice.

**Discussion**

In order to minimize cisplatin-induced ototoxicity, it is vital to find an appropriate strategy to prevent auditory function loss or restore auditory function (Tang, Wang et al. 2021). Cisplatin exposed HEI-OC1 cells, cochleae explant, and mice are utilized in this investigation, and we confirm that agmatine alleviates cisplatin-induced ototoxicity with up-regulated PI3K/AKT signaling pathway. Agmatine supplement may help to reduce cisplatin-induced ototoxicity in clinic.

Cisplatin can transport into cochlea cells and retain for months to years to undergo hydrolysis to form highly reactive aqua cisplatin complexes, which can induce hair cells apoptosis, inflammation, and permanent hearing loss (Rybak, Mukherjea et al. 2019). Our results demonstrate the beneficial effect of agmatine pre-treatment on cisplatin-induced cochleae cells apoptosis with the inhibition of the downstream mitochondrial apoptotic pathway, thereby protecting cochleae cells from cisplatin-induced ototoxicity in the acute
phase. A long-term effect of agmatine administration should be performed in future investigations.

Cisplatin-induced ototoxicity usually appears in the early stages after exposure to cisplatin, primarily affecting the high frequencies and leading to progressive, permanent, and cumulative hearing loss. As indicated in previous reports, DPOAE (Breglio, Rusheen et al. 2017) and ABR (Rybak, Husain et al. 2000) are dysregulated in cisplatin-affected mice. Our investigation testifies that agmatine could improve the degenerative auditory responses ranging from 4 kHz and 32 kHz.

Multiple intracellular signaling pathways, including PI3K/AKT pathway, can phosphorylate Bad (serine-136) to inhibit apoptosis (Wang and Youle 2009, Lu and Imlay 2021, Muri and Kopf 2021). Our present study demonstrates that agmatine could increase phosphorylation of PI3K and AKT diminished by cisplatin exposure. The activation of PI3K/AKT may contribute to the anti-ototoxicity effect of agmatine on cisplatin exposure.

The antioxidant effect of agmatine may act as a scavenger against ROS in human neuronal-like SH-SY5Y cells to maintain mitochondrial membrane potential (Condello, Currò et al. 2011). In RAW 264.7 cells, agmatine has antioxidant activity against lipopolysaccharides-induced ROS accumulation via the activation of PI3K/Akt pathway (Chai, Luo et al. 2016). It is worth noting that agmatine has anti-inflammatory effects, effectively inhibiting the transcription factor NF-κB (Li, Zhu et al. 2020). As to the safe dose identified in our study (200 µM agmatine), a preprint paper indicates that the safe dose of agmatine can reach to 10 mM (Park, Lee et al. 2020). Such discrepancy may need further detailed analysis.
In summation, we demonstrate that agmatine significantly affects the protection against cisplatin-induced ototoxicity by inhibiting ROS production and mitochondrial apoptosis. Our findings further indicate that agmatine can function as a therapeutic or preventive agent in cisplatin-induced ototoxicity.

Conclusions
Agmatine can be utilized to alleviate cisplatin-induced ototoxicity with up-regulated PI3K/AKT signaling.

Declaration of conflict of interest
None.

References


Figure legends

Figure 1. The viability of HEI-OC1 cells with the treatment of designed concentration of cisplatin (a), agmatine (b) and different concentration of agmatine and 30 μM cisplatin for 24 hours. Data were represented as mean ± SD. n = 3. *P < 0.05, **P < 0.01 compared with control group; #P < 0.05, ##P < 0.01 compared with 30 μM cisplatin only group.

Figure 2. The intracellular level of reactive oxygen species (ROS) in HEI-OC1 cells was detected with 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) staining. a, represent fluorescent images from different groups. b, fluorescence intensity was measured with Image J software. Data were represented as mean ± SD. n = 3. **P < 0.01, ***P < 0.001 compared with control group; ##P < 0.01 compared with cisplatin group. Scale bar = 50 μm.

Figure 3. Effect of agmatine on the Bcl-2 family proteins expressions and PI3K/AKT signaling activation in HEI-OC1 cells. Representative Western blot images (a) and quantitative analysis of Bax (b), Bcl-2 (c), p-PI3K (d), and p-AKT (e). Data were represented as mean ± SD. n = 3. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control group; ##P < 0.01 compared with cisplatin group.

Figure 4. Effects of agmatine on cisplatin-induced hair cell loss (a) and ROS level (c) in the middle turns of the cochleae explant. Quantification of myosin 7a-positive (b) and DCFH-DA-positive (d) hair cells. Scale bar = 20 μm. Data were represented as mean ±
SD. n = 6 for each group. *P < 0.05, **P < 0.01 compared with control group; #P < 0.05, ##P < 0.01 compared with cisplatin group.

Figure 5. Effect of agmatine on the Bcl-2 family proteins expression and PI3K/AKT signaling pathway activation in the middle turns of the cochlea explant. Representative Western blot images (a) and quantitative analysis of Bax (b), Bcl-2 (c), p-PI3K (d), and p-AKT (e). Data were represented as mean ± SD. n = 3. **P < 0.01, ***P < 0.001 compared with control group; ##P < 0.01, ###P < 0.001 compared with cisplatin group.

Figure 6. Agmatine prevents auditory function loss in cisplatin exposed mice. ABR (a) and DPOAE (b) thresholds were analyzed. Data were represented as mean ± SD. n = 6 for each group. *P < 0.05, **P < 0.01 compared with control group; #P < 0.05 compared with cisplatin group.
a) Western blot analysis showing protein expression levels of Bax, Bcl-2, p-PI3K, PI3K, p-AKT, and AKT in Control, Agmatine, Cisplatin, and Cisplatin + Agmatine groups. Relative expression levels of Bax, Bcl-2, p-PI3K, PI3K, p-AKT, and AKT are presented in graphs (b-e).

b) Graph showing relative Bax expression levels.

c) Graph showing relative Bcl-2 expression levels.

d) Graph showing relative p-PI3K expression levels.

e) Graph showing relative p-AKT expression levels.