Original Article

Ototoxic effects of mefloquine in cochlear organotypic cultures

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Abstract Mefloquine is a widely used anti-malarial drug. Some clinical reports suggest that mefloquine may be ototoxic and neurotoxic, but there is little scientific evidence from which to draw any firm conclusion. To evaluate the ototoxic and neurotoxic potential of mefloquine, we treated cochlear organotypic cultures and spiral ganglion cultures with various concentrations of mefloquine. Mefloquine caused a dose-dependent loss of cochlear hair cells at doses exceeding 0.01 mM. Hair cell loss progressed from base to apex and from outer to inner hair cells with increasing dose. Spiral ganglion neurons and auditory nerve fibers were also rapidly destroyed by mefloquine in a dose-dependent manner. To investigate the mechanisms underlying mefloquine-induced cell death, cochlear cultures were stained with TO-Pro-3 to identify morphological changes in the nucleus, and with carboxyfluorescein FAM-labeled caspase inhibitor 8, 9 or 3 to determine caspase-mediated cell death. TO-Pro-3 -labeled nuclei in hair cells, spiral ganglion neurons and supporting cells were shrunken or fragmented, morphological features characteristic of cells undergoing apoptosis. Both initiator caspase 8 (membrane damage) and caspase 9 (mitochondrial damage), along with executioner caspase 3, were heavily expressed in cochlear hair cells and spiral ganglions after mefloquine treatment. These three caspases were also expressed in support cells, although labeling was less widespread and less intense. These results indicate that mefloquine damages both the sensory and neural elements in the postnatal rat cochlea by initially activating cell death signaling pathways on the cell membrane and in mitochondria.

Keywords Mefloquine, ototoxicity, neuropathology, outer hair cell, inner hair cell, spiral ganglion, neurons, axon degeneration

Introduction

Malaria is a leading cause of death and disease worldwide, especially in developing countries. Approximately 300 to 500 million people develop malaria and 1.5-3 million, mostly children, die each year [1-3]. Mefloquine (Lariam®) is one of the most widely used antimalarial drugs. Because of its long half-life, mefloquine is often used at moderate, prophylactic doses by travelers and military personnel. The typical prophylactic dose for chloroquine resistant faliparum malaria is 250 mg/week [4]. For severe cases of drug resistant ma - laria, the therapeutic dose of mefloquine in the blood is up to 10 times higher than with prophylaxis [5-6].

With the increased use of mefloquine and higher dosing have come increased reports of adverse effects. Among malarial patients, the risk of adverse effects runs at approximately 1:1200[7]; however, other studies report the risk of adverse events to be in the range of 12-90% [8]. The neurological effects of mefloquine typically occur within 72 h of treatment and are more common in women than men [7]. Common adverse effects include anxiety, panic attacks, nightmares, dizziness, tremor,

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headache, fatigue, grand mal, and suicidal ideation [7, 9–16]. When subjective criteria are used, 11–17% of travelers report being incapacitated by mefloquine. Milder CNS disturbances (dizziness, headaches, insomnia) are seen in up to 25% of individuals with prophylactic doses and up to 90% with therapeutic doses [7, 17]. Hearing and balance disturbances have been reported after mefloquine treatment. One study reported high frequency hearing loss and tinnitus in 3 patients treated with mefloquine, with 2 of the 3 patients showing long term hearing loss and tinnitus following treatment [18]. In 22 normal healthy volunteers treated with therapeutic doses of mefloquine (750 mg and 500 mg) [10], all developed severe side effects. The most common complaint was vertigo which was experienced by 96% of subjects, with 73% developed severe grade 3 vertigo that required bed rest and medication. Collectively, these results suggest that prophylactic or therapeutic doses of mefloquine may affect the inner ear. However, we are unaware of any systematic investigation of the potential ototoxic effects of mefloquine on the inner ear. To address this issue, we treated cochlear organotypic cultures from postnatal day 3 (P3) rats with various doses of mefloquine to evaluate its toxic effects on the sensory hair cells and spiral ganglion neurons.

Methods

Cochlear organotypic cultures and isolated spiral ganglion cultures: P3 F344 rats were used for this study (Charles River Laboratories). The rat pups were decapitated and the cochlea removed as described previously [19–20]. The cochlear basilar membrane was microdissected out in Hanks Balanced Salt Solution (HBSS, Invitrogen) and placed on a droplet (15 μl) of freshly made rat-tail collagen gel on the bottom of a 35 mm tissue culture dish. The collagen gel contained rat tail collagen, 10X basal medium eagle (BME) medium and 2% sodium carbonate in a ratio of 9:1:1. The cochlear explants were incubated in 1.2 ml of serum free medium (1X BME plus serum-free supplement, 1% bovine serum albumin, 2 mM glutamine and 20% glucose) in a CO_2 incubator at 37℃ overnight, and then treated in 2 ml culture medium containing various doses of mefloquine for 24 h.

For isolated spiral ganglion cultures, the cochlear basilar membrane containing the organ of Corti and spiral ganglion was dissected out and then the spiral ganglion in Rosenthal's canal was separated from the basilar membrane. The spiral ganglion neurons were triturated by a fire-polished Pasteur pipette in a small test tube containing 500 μl DMEM/F-12 with 10% FBS. The suspension of dissociated spiral ganglion neurons was seeded at a concentration of 100–200 cells/mm2 in a 35 mm culture dish that had been pre-coated with polylysine (500 μg/ml) and laminin (20 μg/ml). Spiral ganglion cultures were stored in an incubator at 37 oC with 5% CO_2 overnight. On the second day, spiral ganglion cultures were treated with various doses of mefloquine for 24 h.

Mefloquine treatment: Cochlear organotypic cultures were treated for 24 h with 7 different doses of mefloquine (n=10/group): 0 μM (Control), 10 μM, 25 μM, 35 μM, 50 μM, 100 μM and 200μM. The isolated spiral ganglion cultures were treated with 50 μM mefloquine for 24 h.

Sample preparation: At the end of the experiment, cochlear organotypic cultures and isolated spiral ganglion cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and stained as described below. Afterwards, the cochlear basilar membrane or spiral ganglion cultures were mounted in glycerin on a glass slides and the cover slipped on.

Staining: After fixation, the specimens were rinsed in 0.1 M phosphate buffered saline (PBS). For staining the stereocilia and cuticular plate of the hair cells, the fixed specimens were incubated in 0.25% Triton X100 for 5 minutes and then immersed in TRITC-labeled phalloidin (Sigma P1951, 1:200) or FITC-conjugated phalloidin (Sigma P-5282, 1:200) in PBS for 30 minutes. Specimens were rinsed three times in 0.1 M PBS and mounted in glycerin on glass slides.

For staining spiral ganglion neurons and auditory nerve fibers, the fixed specimens were rinsed in 0.1 M PBS incubated for 2 h in a blocking solution consisting of 10% normal goat serum and 10% Triton X-100 in 0.1 M PBS. Afterwards, specimens were immersed in a solution containing 20 μl of mouse anti-neurofilament 200 antibody (Sigma N0142, clone N52), 20 μl Triton