Pharmacological Reports 67 (2015) 317–325

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original research article

Neurotrophic and antioxidant effects of silymarin comparable to 4-methylcatechol in protection against gentamicin-induced ototoxicity in guinea pigs

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ARTICLE INFO

Article history:
Received 21 August 2014
Received in revised form 14 September 2014
Accepted 6 October 2014
Available online 22 October 2014

Keywords:
Gentamicin
Ototoxicity
Silymarin
Neurotrophic factors
Antioxidants

ABSTRACT

Background: Despite that gentamicin is a very effective aminoglycoside, its potential ototoxicity which is of irreversible nature makes a challenge and limitation for its use. This study was designed to investigate possible neurotrophic and antioxidant effects of silymarin comparable to 4-methylcatechol in protection against gentamicin-induced ototoxicity.

Methods and results: Twenty pigmented guinea pigs were divided into four equal groups, where group I served as normal control group. The other groups received gentamicin (120 mg/kg/day, *ip*) for 19 days where group II given vehicle of 1% CMC, group III and group IV were pre-treated 2 h before gentamicin by 4-methylcatechol (10 μg/kg, *ip*) and silymarin (100 mg/kg, oral gavage), respectively. The main findings indicated that silymarin exhibited restoration of nerve growth factor (NGF) levels and increased tropomyosin-related kinase receptors-A (Trk-A) m-RNA expression in cochlear tissue and preservation of hair cells of organ of Corti by scanning electron microscopy (SEM) with significant decrease in auditory brainstem response (ABR) threshold compared to 4-methylcatechol. Only silymarin caused significant amelioration in oxidative stress state by reducing malondialdehyde (MDA) levels and increasing catalase activity.

Conclusions: Silymarin exerts superiority over 4-methylcatechol when recommended as protective agent against gentamicin ototoxicity based on its efficient neurotrophic and antioxidant activities.

Introduction

Gentamicin still has a considerable position in treatment of infections caused by Gram-negative bacteria due to its low cost and high efficacy even in some resistant cases. It has favorable pharmacodynamic features such as the concentration-dependent bactericidal activity with a unique post-antibiotic effect and the strong synergism with other antibiotics such as vancomycin and β-lactams [1,2]. But unfortunately, gentamicin causes nephrotoxicity and ototoxicity that limit its use [3]. Meanwhile, nephrotoxicity is generally mild and reversible, on the reverse; ototoxicity is irreversible owing to drug-induced apoptosis of auditory and vestibular sensory cells resulting in permanent hearing impairment [4]. Basically, gentamicin should be given in a full-dose to avoid possibility of bacterial resistance and provide effective therapy. It has fast access to perilymph within 60–90 min after parenteral administration and remains detectable in hair cells up to 4 months after exposure and the overall ototoxic hearing loss occurs in a concentration-dependent manner [5,6]. Usually hearing loss course occurs within days to weeks to reach its maximal threshold shift and is often of bilateral presentation [7]. Gentamicin ototoxicity is a deceiving condition, since the high-frequency hearing is lost first, thus the patient may be unaware of it. Also, the deafness may occur even after therapy is discontinued [2,8]. This makes the true incidence is often underestimated and when extended high frequency testing was performed, hearing loss was reported in 47% patients with a history of aminoglycoside

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http://dx.doi.org/10.1016/j.pharep.2014.10.007
1734-1140/

treatment [9]. Gentamicin is a cationic aminoglycosides that internalized the cell by endocytosis where it forms a complex with iron [10]. This iron–gentamicin complex enhances iron-catalyzed oxidations and directly promotes formation of reactive oxygen species (ROS) [11,12]. ROS have been detected in the sensory epithelium after aminoglycoside administration [13] and found to induce molecular pathways responsible for disruption of the survival of hair cells and spiral ganglion neurons in the auditory portion of the inner ear [14,15]. The contribution of neurotrophic growth factors in preventing aminoglycosides ototoxicity suggests involvement of the auditory nerve [16]. Previously, it was believed that neuronal damage is of irreversible nature, but recently it has become apparent that damaged neurons could regenerate in an active process under influence of neurotrophic factors [17]. There are many neurotrophic factors and their relevant receptors that studied in context of neuronal development and survival. Among them is the nerve growth factor (NGF) that interacts with tropomyosin-related kinase receptors-A (Trk-A), evoking signaling events that can lead to changes in gene transcription and regulate neuronal survival [18–20]. Despite introduction of neurotrophins themselves as regenerative treatment of various nervous system disorders [21–23], they face significant obstacles such as the short half-life range from few minutes to just a few hours owing to their peptide structure and enzymatic degradation [24,25] as well as their complex and costly production process that represents a barrier to use them commercially [26]. Therefore, proposal of a pharmacological agent of non-peptide nature to enhance the neuro-regenerative pathways can potentially overcome these limitations and generate valuable insight into regulation of cochlear neuron survival and re-growth [27]. Silymarin is the active principle purified from the fruit of *Silybum marianum* that used so far mostly as a hepatoprotective and/or antioxidant agent [28]. Recently, silymarin received attention due to its neuroactive and neuroprotective activities that extensively investigated in many neurological disorders [29–31]. In this context, it is reasoned to investigate the possible neurotrophic and antioxidant effects of silymarin comparable to 4-methylcatechol in protection against gentamicin ototoxicity induced in guinea pigs.

Materials and methods

Drugs and chemicals

Gentamicin sulfate (80 mg/2 ml ampoule) was obtained as a product of Memphis Co. for Pharm. & Chem. Ind. Cairo-A.R.E. under authority of Schering-Plough Corporation, USA. Silymarin (micronized, 140 mg capsule) was obtained as a product of Medical Union Pharmaceuticals, MUP, Egypt. 4-methylcatechol, 95+% was obtained from Sigma–Aldrich Chemical Co., Germany. Ketamine hydrochloride injection USP (Ketamax, 50 mg/ml) was obtained as a product of Troikaa Pharmaceuticals Ltd., India. Other chemicals are of analytical gradient and were obtained from Sigma–Aldrich Chemical Co., unless indicated otherwise.

Animals groups, treatment protocol, and samples collection

Twenty pigmented guinea pigs weighing 400–550 g were used in this experiment based on the anatomical and physiological similarities with the human auditory system [32]. In context, guinea pigs showed no gender differences in susceptibility to aminoglycosides ototoxicity [33]. All animals were in free access to water and food and allowed 1 week of acclimatization and then divided randomly into four equal groups, where group I served as normal control group receiving a vehicle of saline. Group II received gentamicin in a dose of 120 mg/kg/day by intraperitoneal (*ip*) injection for 19 days [34]. Both of group I and group II were given a vehicle of 1% carboxy methylcellulose (CMC). The animals were pre-treated 2 h before gentamicin by either 4-methylcatechol in group III (10 μg/kg by *ip* injection; dissolved in isotonic saline as 10 μg/ml solution) [35,36] or silymarin in group IV (100 mg/kg by oral gavage; prepared as 35 mg/ml solution in 1% CMC [37]. Silymarin pre-treatment was optimized to be 2 h prior to gentamicin administration, based on its reported rapid absorption and tissue distribution with a peak plasma concentration is achieved within 4 h after oral administration [38]. The cochlear function was assessed at the start and after completion of the experiment by auditory brainstem response (ABR) audiometry. At the end of the experiment; the animals were anesthetized with ketamine (40 mg/kg, *ip*) and blood samples were obtained, centrifuged at 3000 × *g* for 10 min to obtain serum samples that preserved at −20 °C for further assay of nerve growth factor (NGF) levels, malondialdehyde (MDA) levels, and catalase activity. Following decapitation, both temporal bones were removed. Both cochleae of each animal were dissected and the left cochlea was frozen immediately in liquid nitrogen for further assay of Trk-A expression. The right cochlea was processed immediately for scanning electron microscope (SEM) examination. All animal experimentation was conducted in accordance to EU Directive 2010/63/EU for animal experiments to minimize animal suffering.

Monitoring of hearing thresholds by auditory brainstem response (ABR) audiometry

Auditory brainstem response (ABR) audiometry was conducted while the animals were under anesthesia with ketamine (40 mg/kg, *ip*), using Smart-EPs of Intelligent Hearing System (IHS). This was done through two-channel recording using three disposable electrodes applied according to the Smart-EP manual specification as the following sites: high frontal Fz (positive electrode) and two electrodes were placed on the left and right mastoids as negative or ground electrode according to the tested ear. All electrodes were connected to the pre-amplifier of the Smart-EP equipment.

ABR was recorded ipsilaterally in response to click stimuli presented at 90 dBnHL and when an identifiable and repeatable ABR response was recorded, the intensity was reduced in 10 dB steps until reaching threshold. Threshold is defined as the lowest intensity at which a recognizable ABR response is recorded. ABR was recorded using alternating polarity, 19.3 s^{-1} repetition rate, filter setting of 150–1500 Hz, gain factor was 50,000 and time window was 0–10 ms. Click stimuli were delivered *via* ER3A-insertphone. The absolute latencies of wave I, III and V, and interpeak latencies (IPLs) of I–III, III–V and I–V were calculated.

Enzyme-linked immunosorbent assay (ELISA) of serum NGF levels

Serum levels of NGF were estimated by sandwich ELISA kit for guinea pig following the manufacturer's protocol (Uscn Life Science Inc., Wuhan, Catalog. No.: E90105Gu) using a 96-well microtiter plate reader. The color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of NGF in the samples is determined by comparing optical density (O.D.) of the samples to the standard curve. The results were expressed as ng/ml.

TrkA mRNA expression in cochlear tissue by real time-PCR

The left cochlea was dissected in an RNA stabilization reagent (RNAlater, Qiagen). Total RNA extraction from cochlear tissue was performed using 1 ml of Trizol reagent per 50 mg of tissue (Invitrogen, USA) following manufacturer's instructions. The yield of total RNA was quantified spectrophotometrically. A total of 1 μg