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Mitochondria-Targeted Antioxidant SkQR1 Ameliorates Gentamycin-Induced Renal Failure and Hearing Loss

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Abstract—The influence of the mitochondria-targeted antioxidant SkQR1 on gentamycin-induced nephrotoxicity and ototoxicity has been analyzed. SkQR1 reduces the death of kidney epithelium cells and decreases the severity of renal failure caused by gentamycin application and also lowers the animals' mortality. Treatment with SkQR1 also decreases gentamycin-induced hearing loss. Mitochondria-targeted antioxidants, such as SkQR1, are new promising agents for preventing negative consequences of therapy with antibiotics.

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Key words: kidney, organ of Corti, mitochondria, antioxidant, nephrotoxicity, ototoxicity, antibiotics

Beginning from the discovery of streptomycin and up to the present, aminoglycoside antibiotics are used as effective agents for treatment of infections, mainly those caused by gram-negative microorganisms and having no alternative in the therapy of infections caused by highly resistant strains [1]. Gentamycin is one of the most often used medicals of this class. However, clinical implementation of gentamycin, similarly to the use of other aminoglycoside antibiotics, is strongly limited by its side effects, first of all by very pronounced nephrotoxicity [2] and ototoxicity [3]. Acute disorders in the excretory function of kidneys are observed in about one fourth of patients treated with gentamycin [2], while hearing is irreversibly decreased in 30% of patients [4]. So far there is no real approach for preventing aminoglycoside toxicity, and only a strict dosage of these antibiotics and monitoring of hearing and kidney functions can somewhat lower the risk of adverse effects [4].

Epithelium of proximal renal tubules [5] and external auditory hairs of the organ of Corti [6] are, in particular, targets of gentamycin toxicity [6]. The death of these cells leads to functional failure of kidneys and of the inner ear. Notwithstanding differences in the structure of these tissues, based on experimental data it is supposed that in these two cases we deal with a similar mechanism of damage, which is associated with induction of oxidative stress [2, 3].

Nephrotoxicity and ototoxicity are the most serious side effects of aminoglycoside antibiotics, especially gentamycin, and this significantly limits their use in medicine. The deleterious effect of gentamycin on kidney and inner ear cells is mainly caused by damage to mitochondria that can be explained by a significant biochemical similarity of mitochondria and bacterial cells. The mitotoxic action of gentamycin is known to be associated with induction of oxidative stress, which leads to cell death and organ dysfunction.

Under conditions of oxidative stress, mitochondria can be the main source of reactive oxygen species (ROS) and also the most ROS-sensitive cell compartment. Thus, mitochondria need special antioxidant protection, because they are a crucially important energetic machine and center of induction and promotion of many signaling pathways [7, 8]. Recent study [9] has revealed that dysfunction of mitochondria is the earliest index of gentamycin-induced nephrotoxicity, preceding morphological and functional changes [9]. The *in vitro* studies on the gentamycin ototoxicity mechanism also reveal the key role of mitochondria in this process [10, 11].

Abbreviations: AKI, acute kidney injury; ROS, reactive oxygen species; SkQR1, 10-(6′-plastoquinonyl) decylrhodamine 19.

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Thus, compounds preventing the development of oxidative stress and normalizing functions of mitochondria can be universal protective agents against gentamycin-induced nephro- and ototoxicity. Mitochondria-targeted antioxidants of the SkQ group [12] developed on the basis of penetrating cations [13] can be highly promising. In our earlier studies the compound SkQR1 was shown to effectively prevent the development of renal failure under conditions of kidney ischemia/reperfusion and myoglobinuria, i.e. conditions associated with an increased production of ROS in kidney cells [14]. In the present work, we have enlarged the spectrum of protective effects of SkQR1 onto the gentamycin-induced damage of kidney and inner ear as another pathologies mediated by oxidative stress and dysfunction of mitochondria.

MATERIALS AND METHODS

The *in vivo* modeling of gentamycin toxicity. Experiments were performed on outbred white male rats (250-450 g) on *ad libitum* diet. The rats were randomly divided into two groups, each including 17 animals: rats from the "gentamycin" group were injected intraperitoneally with gentamycin (KRKA, Slovenia) at the dose of 160 mg/kg daily for six days; rats from the group "gentamycin + SkQR1" were injected intraperitoneally with SkQR1 (100 nmol/kg daily) 3 h before the gentamycin injections. On the next day after the cessation of the gentamycin treatment blood samples were taken from the tail vein of the animals. Concentrations of creatinine and urea were determined in the sera using a CellTac blood analyzer (Nihon Kohben, Italy). Survival of the animals was monitored during 14 days after exposure.

Assessment of hearing acuity. The hearing acuity of the animals was assessed using the Preyer reflex on the 1st, 4th, 7th, and 14th days after the cessation of gentamycin injections. The researcher imposed three standard sounds with different intensity at approximately the same frequency, placing the sound source at the same distance from the rats. The Preyer reflex – a turn of the animal's conch or head towards the sound source – was recorded. The hearing acuity was assessed semi-quantitatively: level 0 corresponded to absence of the reflex in response to all three sounds (nearly absolute deafness); level 1 corresponded to the reflex only in response to the strongest sound (severe hearing reduction); level 2 corresponded to the reflex in response to the strongest and moderate sound but its absence in response to the weakest sound (moderate hearing decrease); level 3 corresponded to the reflex recorded in response to all three sounds (hearing acuity comparable to that of intact animals).

The *in vitro* modeling of gentamycin toxicity. Cultures of kidney tubular epithelium were prepared as described earlier [15]. The cells were cultured in a CO_2-incubator for 1-2 days before experiment in DMEM/F-12 medium (1 : 1) supplemented with 10% fetal calf serum. Before treatment with gentamycin, the cells were incubated for 24 h with SkQR1 at the concentration of 10, 50, and 100 nM. Then the medium was replaced by serum-free medium containing different concentrations of gentamycin, and the cultures were incubated for 24 h. Cell viability was assessed using the standard MTT test. The micro-cultivation was performed in 96-well plates.

RESULTS

Effect of SkQR1 on gentamycin-induced kidney injury. The injections of rats with gentamycin caused a pronounced nephrotoxic effect resulting in development of acute kidney injury (AKI). The daily injections of gentamycin at the dose of 160 mg/kg during 6 days caused disorders in kidney functions that were manifested by 3.5-fold increase in the blood concentration of creatinine compared to the control animals (from 60.8 ± 2.03 to 220.41 ± 41.97 μM) on the 1st day after the cessation of the antibiotic injections. Intraperitoneal injections of SkQR1 at the dose of 100 nmol/kg 3 h before each injection of gentamycin decreased the serum creatinine concentration on the 1st day to 153.94 ± 27.03 μM. Thus, the mitochondria-targeted antioxidant ameliorated the toxic effect of gentamycin (Fig. 1).

Effect of SkQR1 on survival of animals with gentamycin-induced AKI. AKI developed as a result of gentamycin action was life-threatening for the experimental animals. Mortality was monitored during the first 2 weeks after the cessation of gentamycin injections, and the survival of gentamycin-treated animals during this period was 64.7%. On the combined treatment with SkQR1 and gentamycin, the survival was significantly increased to 82.3% on the 14th day (Fig. 2).

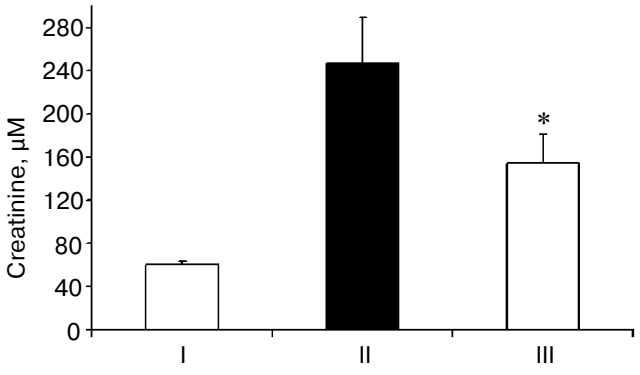

Fig. 1. Concentration of creatinine in the control animals (I), after injections of gentamycin (II), and after injections of SkQR1 before the gentamycin injections (III). Data are presented as the mean ± SEM; * $p < 0.05$ relatively to gentamycin without SkQR1.

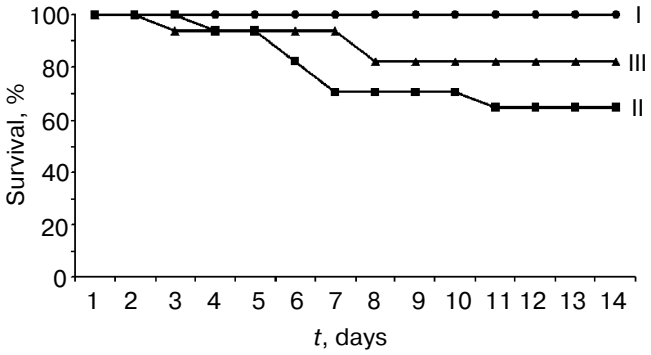

Fig. 2. SkQR1 decreases the mortality of animals as a result of gentamycin-induced acute renal failure. I, control; II, gentamycin; III, gentamycin + SkQR1.

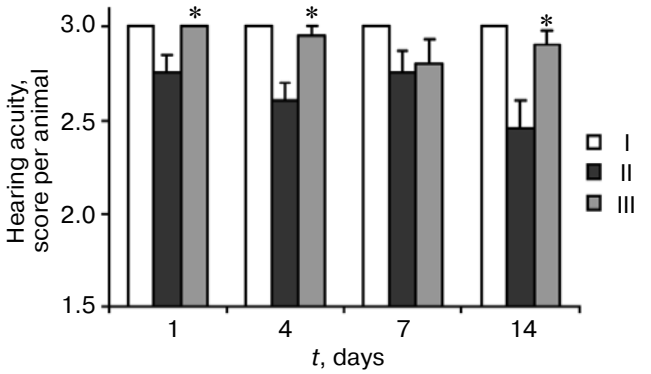

Fig. 3. SkQR1 ameliorates manifestations of the gentamycin-induced hearing loss. I, control; II, gentamycin; III, gentamycin + SkQR1. Data are presented as the mean ± SEM; * $p < 0.05$ relatively to gentamycin without SkQR1.

Effect of SkQR1 on gentamycin-induced decrease in hearing. The protocol used for the gentamycin injections allowed us to assess not only a nephrotoxic effect of the antibiotic, but also its ototoxic effect. Similarly to the kidney damage, the toxic effect of gentamycin on the inner ear developed rapidly. Already on the 1st day after the cessation of gentamycin injections a hearing impairment was recorded in 17.6% of the animals. The number of animals with hearing impairments increased with time, being 25, 33.3, and 27.3%, respectively, on the 4th, 7th, and 14th days after the cessation of the gentamycin injections. Also, the hearing acuity loss increased with time: on the 1st day the score was 2.71 ± 0.17 per animal; on the 4th day it was 2.63 ± 0.18 per animal; on the 7th day it was 2.67 ± 0.19 per animal; and on the 14th day it was 2.45 ± 0.31 per animal (Fig. 3).

SkQR1 (100 nmol/kg) injected 3 h before each injection of gentamycin for 6 days ameliorated the ototoxic effect of the antibiotic, although it did not prevent it. The used protocol of SkQR1 treatment caused lower inner ear damage: on the 1st day after the cessation of the antibiotic injections in the animals injected with both SkQR1 and gentamycin no hearing impairments were observed, and on the 4th day the impairments were observed in 6.2% of animals compared to 25% in the animals injected with gentamycin only. On the 14th day the hearing impairments were recorded in 14.3% of the animals treated with both gentamycin and SkQR1, and the loss in hearing acuity was less pronounced than in the animals treated with gentamycin only (hearing acuity score on the 14th day was 2.45 ± 0.31 per animal in gentamycin-treated rats and 2.86 ± 0.09 per animal in rats treated with both gentamycin and SkQR1) (Fig. 3). Thus, SkQR1 slowed down the development and the level of gentamycin-induced hearing impairments.

Effect of SkQR1 on the gentamycin-induced death of kidney epithelium cells *in vitro*. The assessment of kidney epithelium cells viability by the MTT test revealed that incubation with gentamycin caused cell death during 24 h even at the antibiotic concentration of 0.6 mg/ml, and it reached 80-90% relative to the control at the gentamycin concentration of 5-10 mg/ml. Preincubation with 10 nM SkQR1 for 24 h did not influence the gentamycin-induced death of kidney cells. Preincubation with 50 nM SkQR1 for 24 h significantly increased the survival of kidney cells, from 78 ± 7 to 90 ± 5% ($p < 0.05$) and from 66 ± 5 to 78 ± 4% ($p < 0.05$) at the gentamycin concentrations of 0.6 and 1.2 mg/ml, respectively. Preincubation with 100 nM SkQR1 increased the kidney cell survival from 66 ± 5 to 78 ± 4% ($p < 0.05$) at the gentamycin concentration of 1.2 mg/ml. At higher concentrations of gentamycin the preincubation with SkQR1 did not have a protective effect.

DISCUSSION

In the present work, we studied the possibility of preventing gentamycin-induced renal failure and hearing loss using the mitochondria-targeted antioxidant SkQR1. In this study, we used a conventional model of long-term treatment of animals with high doses of gentamycin [5]. This model clearly demonstrates the development of apoptosis of kidney tubular cells [5] accompanied by mitochondria damage [9] apparently caused by oxidative stress [16] and resulting in acute renal failure of the animals [2]. Concurrently with the damage of the kidney epithelium, gentamycin causes damage to the organ of Corti sensor cells [6, 17]. Disorders in kidney functions is reversible due to regeneration of the kidney epithelium, but the death of hair cells which is irreversible leads to permanent loss of the ability to perceive sounds of certain frequencies [18].

We injected SkQR1 daily during 6 days, 3 h before the injections of gentamycin. This interval was chosen based on the pharmacokinetics of SkQR1: its accumulation in renal tubules starts just 3 h after its intraperitoneal

injection [19]. Moreover, we have shown earlier that SkQR1 injection by this protocol can prevent gentamycin-caused decrease in erythropoietin synthesis in the kidney [14]. In the present work, we have shown that preliminary injections of SkQR1 at the dose of 100 nmol/kg before the injections of gentamycin effectively prevent the development of AKI caused by the treatment with gentamycin. The injections of SkQR1 during the treatment with gentamycin also significantly decreased both acute ototoxicity and subsequent hearing loss after the cessation of the antibiotic injections. Moreover, SkQR1 increased the survival of animals within two weeks after the cessation of SkQR1 and gentamycin injections.

We suppose that the nephro- and ototoxicity of gentamycin are based on similar mechanisms. The antibiotic induces hyperproduction of ROS in mitochondria of the cells (which is widely accepted [9, 20]), and this results in mitochondrial permeability transition pore (PTP) induction, which in its turn leads to apoptotic or necrotic cell death. Subsequently, the loss of functioning cells of the organ becomes the cause of renal dysfunction or of hearing impairments. Such sequence of events is consistent with data of work [16] describing an increased production of hydrogen peroxide in renal mitochondria under the influence of gentamycin. It was shown [9] that intraperitoneal injections of gentamycin to rats resulted in appearance of PTP in the kidney cells, whereas the anti-diabetic drug metformin prevented the development of renal failure and induction of PTP. PTP contribution to *in vitro* gentamycin-induced death of cochlear neurosensor cells was shown [10], and metformin was found to display a protective effect in the ototoxicity model as well [11].

According to this scheme, the protective effect of SkQR1 can be realized in two stages. First, this compound can prevent the hyperproduction of ROS due to a direct antioxidant action [21]. On the other hand, we have earlier shown that SkQR1 induces protective signaling pathways leading to phosphorylation of glycogen synthase kinase 3β and prevention of the induction of PTP [14, 22].

It should be noted that both the nephro- and otoprotective effects of SkQR1 can be also realized by other mechanisms. Thus, we have shown that SkQR1 can either increase the erythropoietin production in the kidney or prevent the gentamycin-induced decrease in its production [14]. Since erythropoietin is an important cytoprotector and anti-ischemic agent [23], its production by the kidney can prevent damage to the kidney tissue itself and to remote organs, in particular, the organ of Corti cells [24]. On the other hand, the prevention by SkQR1 of hearing loss of animals can be directly associated with retention of the kidney excretory function. It has been established that 70-95% of gentamycin is eliminated from the organism in urine, and renal failure, which is increasing with gentamycin use, results in its accumulation in the organism and, consequently, in an increase in its damaging effect on the inner ear [25]. By the way, this seriously limits the use of gentamycin in patients with some forms of renal dysfunction due to the increased risk of hearing loss.

Note that notwithstanding a significant nephroprotective effect of SkQR1 *in vivo*, its action was less profound in the kidney tissue culture. Consequently, the protective effect of SkQR1 on the renal tissue (and, possibly, on the cells of organ of Corti) can be due not only to the direct antioxidant action in the tubular cells, but to the influence of other systems of the organism as well [14, 19].

Thus, the gentamycin-induced production of ROS by mitochondria can initiate some pathological cascades resulting in damage and death of kidney tubular cells and of hair cells of the inner ear that finally results in dysfunction of both organs. Therefore, mitochondria-targeted antioxidants, such as SkQR1 used in the present work, seem to be promising new agents for prevention of negative consequences of antibiotic therapy. The amelioration by mitochondria-targeted antioxidants of nephrotoxicity/ototoxicity of aminoglycoside antibiotics is promising for expanding of the therapeutic window for their use, with respect to doses and duration of treatment, and also to lower risk of their use in children, pregnant women, and patients with renal insufficiency.

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