MELATONINE AND ERYTHROPOIETIN PREVENTS GENTAMICIN INDUCED NEPHROTOXICITY IN RATS

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Abstract

Melatonin (MLT) is an epiphyseal chronobiotic hormone, also known for its antioxidant effects. Besides erythropoiesis, erythropoietin (EPO) possesses other biological functions (neuroprotection, nephroprotection). We focused on the assessment of MLT and EPO nephroprotective effects in a gentamicin (GM) toxicity model. Forty adult male Wistar rats were divided into 5 groups: control, GM, EPO + GM, MLT + GM and MLT + EPO + GM, consisting of 8 rats each. Chemicals were administered for 10 days, daily, by i.p. route. Both MLT and EPO prevented kidney damage reflected by lower urinary N-acetyl-β-d-glucosaminidase (NAG) activity index, reduced kidney structural damage, increased urinary density and decreased blood urea and creatinine concentrations. The best protective effect was provided by MLT and EPO association.

Rezumat

Melatonina (MLT) este un hormon cronobiotic epifizar cunoscut și pentru efectele sale antioxidante. Pe lângă eritropoieză, eritropoietina (EPO) posedă și alte funcții biologice (neuroprotecție, nefroprotecție). Studiul a constatat în evaluarea efectelor nefroprotectoare ale MLT și EPO într-un model experimental de toxicitate cu gentamicină (GM). Patruzeci de șobolani masculi adulți Wistar au fost împărțiți în 5 grupuri: control, GM, EPO + GM, MLT + GM și MLT + EPO + GM, constând din 8 șoareci fiecare. Compușii au fost administrați zilnic, timp de 10 zile, pe cale i.p. Atât MLT cât și EPO au prevenit afectarea renală reflectată de indexul activității N-acetil-β-d-glucozaminidazei (NAG) urinare, o afectare structurală a rinichiului redusă și creșterea densității urinare, precum și scăderea concentrațiilor de uree și creatinină din sânge. Cel mai bun efect de protecție a fost conferit de asocierea dintre MLT și EPO.

Keywords: erythropoietin, melatonin, nephroprotection

Introduction

Gentamicin (GM) is an aminoglycoside broad spectrum antibiotic widely used to treat severe Gram-negative infections, particularly valuable in sepsis. However, its clinical use is limited by its potential ototoxic and nephrotoxic effects [9]. Nephrotoxicity has been related to the selective accumulation of GM in the renal cortex [8], especially in the renal proximal convoluted tubules (50 to 100 times greater than serum) and production of free radicals [3]. Studies performed over the last decades reported that GM induced nephrotoxicity is characterized by tubular necrosis particularly in the proximal tubule. The mechanism of GM toxicity remains unknown. However, it is believed that GM enhances the production of a generation of reactive oxygen species leading to deficiency in intrinsic antioxidant activity. In

consequence the use of substances with high antioxidant activity has been showed to prevent or ameliorate GM nephrotoxicity [1, 16].

Erythropoietin (EPO) is a glycoprotein hormone that controls erythropoiesis. Human EPO has a molecular weight of 34 kDa and can be found in the literature under the name hematopoietin or hemopoietin [22]. It is produced in the kidney by fibroblasts in the immediate vicinity of the proximal convoluted tubules and peritubular capillaries. The production of this hormone also take place in the perisinusoidal cells of the liver; however hepatic production of EPO characterizes predominantly fetal and perinatal life. Besides erythropoiesis, EPO possesses other biological functions (neuroprotection, wound healing, nephroprotection) [21].

Melatonin (N-acetyl-5-methoxytryptamine) (MLT) is the major epiphyseal hormone secreted during the

night in all vertebrates and was discovered by Aaron B. Lerner in 1958 [2]. In addition to the use of MLT as a chronobiotic or hypnotic to influence or restore circadian rhythms, melatonin's antioxidant effects can be used to reduce the risk of kidney damage. There is sufficient evidence demonstrating that oxidative stress contributes to renal lesions and injury [4].

Based on the above-mentioned data, the hypothesis was made that EPO and MLT are major candidates in the prevention and amelioration of GM induced renal injury. Therefore, the main goal of our study was to identify which of the previously mentioned compounds, respectively, the association between the two provides the best nephroprotective effect. This effect was evaluated by measuring renal function parameters such as plasma urea and creatinine concentrations, urinary NAG (N-acetyl- β -D-glucosaminidase) index activity and urinary specific gravity as well as renal histological and urinary cytology studies.

Materials and Methods

Drugs and chemicals

GM - gentamicin sulphate (Gentamicina® 40 mg/mL) was obtained from KRKA Romania; EPO - beta erythropoietin (Neorecormon® 2000 IU) was supplied from Roche Romania; MLT - pure melatonin (Sigma®) was supplied from Redox Romania.

Animals

Forty adult male Wistar rats weighing 250 ± 12 g were selected for this study. The experiments and animals welfare were in accord to Directive 2010/63/EU and national legislation (Law no. 43/2014). All the experimental procedures were approved by the Committee for Bioethics of the University of Agricultural Science and Veterinary Medicine Cluj-Napoca, Romania (UASVM CN) and authorized by state veterinary authority. The animals were caged in polycarbonate cages, at controlled temperature of $21 - 22^{\circ}\text{C}$, humidity (40 - 60%) and 12/12 h light/dark cycle. Standard laboratory animal forage, provided by "Cantacuzino" National Institute for Research and Development Bucharest and water were freely available. The experiment was carried in the Establishment for Laboratory Animals of UASVM CN.

Experimental design

Animals were divided into 5 groups consisting of 8 rats each, the study lasted for 10 days. The rats received therapy daily by i.p. route. In the control group rats were injected with normal saline solution. Group GM was injected with 100 mg GM/kg bw/day. Group EPO + GM was injected with 100 UI EPO/kg bw/day and 100 mg GM/kg bw/day. EPO was administered separately, one hour before GM. Group MLT + GM received 20 mg MLT/kg bw/day dissolved in 5% ethanol [5] and 100 mg GM/kg bw/day. MLT was given separately, one hour before GM. Group

MLT + EPO + GM received 20 mg MLT/kg bw dissolved in 5% ethanol, 100 mg EPO/kg bw/day and 100 mg GM/kg bw/day. The compounds were administered separately: MLT 2 hours before GM administration and EPO one hour before GM administration. All animals were euthanized in the 11th day by isoflurane euthanasia chamber. Blood samples were collected into heparinised tubes and complete blood count was performed. Thereafter plasma was separated by centrifugation at 3000 rpm for 10 minutes at 4°C for the determination of urea and creatinine. Urine was collected by cystocentesis into clean tubes and centrifuged at 1000 rpm for 5 minutes at 4°C . The supernatant was used as the source of experimental product for NAG index testing. The left kidney was collected and fixed with 10% formaldehyde solution at room temperature for histological evaluation [1].

Haematological analysis

Complete blood count (CBC) was performed with the Abacus Junior Vet automatic haematology analyser (Diatron Messtechnik, Hungary). Blood smears were stained using Diff Quick and Giemsa staining. Smears were examined for differential blood count and for erythrocyte morphology in the feathered region of the film, at high power ($\times 100$).

Biochemical Analysis

Determination of plasma urea and creatinine concentrations

Plasma creatinine concentration was measured using a kinetic spectrophotometric fixed-time method at 510 nm (Jaffe reaction), while the plasma urea concentration was also determined using a kinetic spectrophotometric fixed-time method at 450 nm.

Determination of Urinary NAG index concentration

Determination of urinary NAG index activity was done by the following method: urine samples were centrifuged at 1000 rpm for 5 min at 4°C then the enzymatic activity was measured by a spectrophotometric, colorimetric method. NAG index was measured using an end-point spectrophotometric reaction whereas the urinary concentration of creatinine was determined by a spectrophotometric, kinetic reaction using the Jaffe method. Urinary NAG index was calculated by the following equation: NAG index (U/g) = urinary NAG activity (U/L)/urinary creatinine concentration (g/L) [12].

Urinary specific gravity evaluation

Urinary specific gravity was determined by a refractometric method using the Abbe-Zeiss refractometer which was calibrated according to the manufacture's indications.

Histopathological evaluation

A complete necropsy was performed in all cases, and the left kidney was removed for histological analysis. The renal samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Serial, 4 μm thick sections were made using the Leica RM 2125

RT microtome. These sections were stained using the haematoxylin-eosin (HE) method. The slides were examined using an Olympus BX 51 microscope and pictures were taken with an Olympus UC 30 digital camera and processed using the Olympus Stream Basic program.

For the evaluation of histological damage, the degree of degeneration and tubular necrosis, the occurrence of mononuclear cell infiltration and the emergence of the hyaline casts were followed. Histopathological changes were assessed by scores from 0 to 4, where 0 is healthy tissue, 1 represents minimal lesions, 2 moderate lesions, 3 moderate to severe lesions, and 4 severe lesions, according to Randjelovic *et al.* [11].

Statistical Analysis

All data are reported as the mean $\pm$ SEM. To assume Gaussian distribution normality distribution was checked by D'Agostino and Pearson omnibus normality test. One-way analysis of variance ANOVA, followed by Bonferroni's Multiple Comparison test procedure was done for pair-wise comparisons. Pearson's correlation was used in order to assess the correlation, interpretation was done according to Colton scale. Statistical significance was set at $p < 0.05$ (95% confidence interval). Statistical values and figures were obtained using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California, USA. Type I collagen of bovine origin was extracted by the currently

Results and Discussion

All the rats subjected to GM toxicity remained alive until the end of the study. The body weight decreased but no significantly, similarly to the control group. In all groups, complete blood count reflected normal values similar to the control group. Urinary NAG index activity was 2 folds lower in the EPO group (55.7 ± 1.19 U/g) ($p < 0.05$), 3.5 folds lower in the MLT group (32.5 ± 0.60 U/g), with the lowest activity recorded in the MLT + EPO group (30.1 ± 0.50 U/g) when compared with GM group (122.8 ± 3.61 U/g) (Figure 1). Control group recorded normal iNAG values (1.6 ± 0.49 U/g). Urinary specific gravity remained between normal ranges in the EPO, MLT and MLT + EPO groups (1.02 ± 4.81 , 1.03 ± 5.42 and 1.03 ± 6.67) when compared to the GM group (1.01 ± 3.58) (Figure 2).

Blood urea and creatinine levels were found to be decreased in the EPO (creatinine - 4.03 ± 0.66 mg/dL and urea - 124.93 ± 15.67 mg/dL) ($p < 0.001$), MLT (creatinine - 2.43 ± 0.69 mg/dL and urea - 77.15 ± 10.16 mg/dL) ($p < 0.001$) and MLT + EPO (creatinine - 2.33 ± 0.30 mg/dL and urea - 70.05 ± 8.20 mg/dL) ($p < 0.001$) groups when compared to the GM group (creatinine - 11.37 ± 1.28 mg/dL and urea - 324.5 ± 38.27 mg/dL) (Figures 3 and 4).

Figure 1.

Effects of EPO and MLT therapy on iNAG activity in GM induced nephropathy (U/g)
(mean $\pm$ SEM, * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$, 8 animals/group)

Figure 2.

Effects of EPO and MLT therapy on urine specific gravity in GM induced nephropathy
(mean $\pm$ SEM, *** = $p < 0.001$, 8 animals/group)

Figure 3.

Effects of EPO and MLT therapy on blood creatinine levels in GM induced nephropathy (mg/dL)
(mean $\pm$ SEM, ** = $p < 0.01$, *** = $p < 0.001$, 8 animals/group)

Figure 4.

Effects of EPO and MLT therapy on blood urea levels in GM induced nephropathy (mg/dL) (mean $\pm$ SEM, *** = $p < 0.001$, 8 animals/group)

Figure 5.

Effects of EPO and MLT therapy on mononuclear cell infiltration in GM induced nephropathy (semi-quantitative assessment) (mean $\pm$ SEM, ** = $p < 0.01$, *** = $p < 0.001$, 8 animals/group)

Figure 6.

Effects of EPO and MLT therapy on hyaline casts in GM induced nephropathy (semiquantitative assessment) (mean $\pm$ SEM, ** = $p < 0.01$, *** = $p < 0.001$, 8 animals/group)

Figure 7.

Effects of EPO and MLT therapy on tubular degeneration in GM induced nephropathy (semi-quantitative assessment) (mean $\pm$ SEM, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, 8 animals/group)

Figure 8.

Effects of EPO and MLT therapy on tubular necrosis in GM induced nephropathy (semi-quantitative assessment) (mean $\pm$ SEM, * = $p < 0.05$, *** = $p < 0.001$, 8 animals/group)

These findings were confirmed by histopathology where the EPO ($p < 0.05$), MLT ($p < 0.05$) and MLT + EPO ($p < 0.05$) groups scored the lowest in micro structural damage when compared to the GM group (Figures 5, 6, 7 and 8).

The control group maintained normal kidney morphology. GM group revealed massive tubular degeneration, slight mono-nuclear tubular necrosis, slight mononuclear inflammatory infiltrate, mild hyalinosis. EPO + GM group presented severe tubular degeneration and numerous hyaline casts. In the MLT + GM group, mild tubular degeneration

and necrosis, mild hyalinosis of renal tubules were found; MLT + EPO + GM group presented mild tubular

degeneration and necrosis, slight hyalinosis and few hyaline casts (Figure 9).

Figure 9.

Histological changes in different experimental groups.

Control group (A) showing normal morphology; GM group (B and C), Massive tubular degeneration, slight mononuclear tubular necrosis, slight mononuclear inflammatory infiltrate, mild hyalinosis EPO + GM group (D), Severe tubular degeneration, numerous hyaline casts; MLT + GM group (E), Mild tubular degeneration and necrosis, mild hyalinosis of renal tubules; MLT + EPO + GM group (F), Mild tubular degeneration and necrosis, slight hyalinosis, a few hyaline casts; HE x200, Scale bar = 100 µm

In order to assess the renal damage induced by GM toxicity, histopathological changes, urinary specific gravity, blood urea, creatinine and NAG index activity were evaluated. iNAG is a lysosomal enzyme located especially in the renal proximal convoluted tubules epithelia. An increase in urinary NAG enzyme activity strongly suggests tubular cell damage therefore recommending NAG as a renal tubular injury biomarker [6].

There are numerous experimental studies showing that EPO interacts with specific receptors identified in various tissues, resulting in a series of cytoprotective effects such as mitogenesis, angiogenesis, inhibition of apoptosis and promoting reparative vascular events by mobilization of endothelial precursor cells. For example, it has been demonstrated [6, 14] that EPO produced by astrocyte cell cultures can protect cortical neurons from the harmful effects of hypoxia. Moreover, *in vivo* experimental models showed that neutralization of endogenous EPO increased ischemic brain damage [15]. However, the reduced amount and delayed secretion of EPO in the brain suggests limited protective action against hypoxic lesions or toxicity at this level [6, 15, 20]. Since functional receptors for EPO were identified in epithelial tubular cells, endothelial and mesangial cells, the assumption that there might be an important paracrine EPO axis with kidney cytoprotective effects was made.

During the early stages of ischemic acute renal failure, EPO expression is virtually absent, while EPO receptor expression is maintained within normal limits. It has been shown that the administration of exogenous EPO in both *in vivo* and *in vitro* models of experimental hypoxic and ischemic acute renal failure induced significant structural and functional recovery of the kidney [7, 13].

In rat experimental models of induced unilateral and bilateral kidney ischemia-reperfusion injury, treatment with EPO (5000 UI/kg) produced a decrease in apoptosis among the epithelial and tubular cells and an increase of mitotic index. Moreover, serum creatinine was significantly lower in subjects treated with EPO (21 ± 8 µmol/L) compared with controls (40 ± 10 µmol/L). It has been demonstrated that darbepoetin, an analogue of EPO, possesses nephroprotective effects comparable to EPO in bilateral ischemia-reperfusion injury and the beneficial effects were observed with the use of both molecules even if the administration was delayed for up to six hours after the restoration of tissue perfusion. These findings have raised the hypothesis that both alpha darbepoetin and EPO have potential therapeutic applicability and could be used as nephroprotective medication not only for patients at risk for acute renal ischemia but also for those who have already had an episode of renal injury [10, 17].

Other studies have reported similar findings, demonstrated by the histological and functional kidney tissue rehabilitation in experimental models of kidney ischemia-reperfusion injury and Cisplatin induced renal toxicity [14].

MLT is a powerful antioxidant, being a substance that directly neutralizes free radicals $\text{HO}^\cdot$, $\text{O}_2^\cdot$, NO , and indirectly by activating large classes of anti-oxidant enzymes including SOD (superoxide dismutase), CAT (catalase) and GPx (glutathione peroxidase). Unlike other anti-oxidants, MLT cannot be reduced to the initial state after being oxidized because it forms stable compounds in oxidized state. MLT also prevents damage to DNA molecules thus acting as anti-carcinogenic agent. It also reduces Parkinson's injuries, and in mice, MLT increased the life span by 20% [18]. On pinealectomized mice, Warren *et al.* demonstrated that all individuals suffered renal lesions which could be prevented by the administration of exogenous MLT [19].

Conclusions

Our study demonstrates significant differences between the EPO, MLT and MLT + EPO treated groups in contrast with the GM group with the most notable differences in the MLT + EPO group which showed the most intense degree of nephroprotection against GM induced renal damage. Although both EPO and MLT alone demonstrated a proper nephroprotective effect it seems that combined MLT and EPO therapy shows the most promising effects in the prevention and counteraction of renal damage induced by high doses of GM administration.

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