AMLEORATIVE ROLE OF ESCULETIN-MEDIATED RENOPROTECTION AGAINST GENTAMICIN-INDUCED NEPHROTOXICITY AND POSSIBLE INVOLVEMENT OF N-METHYL-D-ASPARTATE RECEPTORS

ALKA BHATIA, RISHMEEN CHADHA, UPENDRA KUMAR JAIN, GURPREET SINGH*
Department of Pharmacy, Pharmacology Division, Chandigarh College of Pharmacy, Landran, Mohali, Punjab, India.
Email: gurpreet.ccp@cgc.edu.in

ABSTRACT

Objective: In this investigation, the amleorative role of esculetin (Esc) was investigated in gentamicin (Genta) nephrotoxicity in rats and the possible role of N-methyl-D-aspartate receptors (NMDAR) in genta-induced nephrotoxicity.

Methods: Genta (100 mg/kg/day, i.p. for 7 days) was administered to rats for the induction of nephrotoxicity, and subsequently, the extent of renal damage was measured by estimating creatinine clearance (CrCl), blood urea nitrogen (BUN), uric acid, microproteinuria and fractional excretion of sodium, and potassium. In addition, renal superoxide anion generation (SAG), thiobarbituric acid reactive substance (TBARS), and reduced glutathione (GSH) level were used to evaluate renal oxidative parameters. Renal myeloperoxidase (MPO) activity was used to measure renal inflammation. D-serine, NMDA agonist was used in this study to evaluate the role of NMDAR antagonist in genta-induced nephrotoxicity. Histopathological examination was performed using hematoxylin and eosin staining method.

Results: Genta-treated rats exhibited remarkable changes in renal parameters like increase in BUN, uric acid, microprotein fractional excretion of sodium and potassium with decrease in CrCl and similarly biochemical parameters like increase in SAG, thiobarbituric acid reactive species (TBARS), MPO activity with decrease in GSH level. Treatment with Esc (5 and 10 mg/kg/day, i.p for 7 days) significantly reduced the genta-induced nephrotoxicity and the protective effect of Esc was confirmed by normalization of tubules but not with the combined use of Esc + genta + D-serine treated rats.

Conclusion: Esc displayed protective effect in genta-induced nephrotoxicity but combined effect of Esc + genta + D-serine abolished the protective effect of Esc thus confirming that NMDAR may be involved in genta-induced nephrotoxicity.

Keywords: Nephrotoxicity, Gentamicin, D-serine, Esculetin, N-methyl-D-aspartate receptor.

INTRODUCTION

Nephrotoxicity occurs when kidney-specific detoxification and excretion do not work properly due to the damage or destruction of kidney function by exogenous or endogenous toxicants [1]. Gentamicin (Genta) is one of the most effective aminoglycoside antibiotics used against Gram-negative infections. However, nephrotoxicity is the major untoward outcome of genta treatment. Recent reports suggest that 30% of the patients treated with genta for more than 7 days develop nonoliguric renal dysfunction and results in apoptosis as well as necrosis of tubular epithelial cells [2,3]. The occurrence of nephrotoxicity with genta is up to 60% in case of patients admitted in intensive care units [4].

N-methyl-D-aspartate receptors (NMDARs) belong to a class of ionotropic glutamate receptors that also include the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors and kainate receptors [5]. NMDAR is glutamate-gated cation channel with high calcium permeability and critical for the development of the central nervous system [6]. NMDARs are also expressed across a wide spectrum of non-neuronal cells, including central and peripheral glial cells, endothelium, kidney, bone, pancreas, and others [5]. NMDAR forms a heterotetramer composed of NR1 and NR2 (A-D) subunits and more rarely, NR3 subunits [7]. The NR1 subunit is the main subunit of the NMDAR essential for channel activity, whereas the NR2 subunits, although not essential for function, can confer modulatory properties [8]. NR1 is present in total rat kidney, cortex, and medulla and out of the NR2 subunits, only the NR2C subunit protein is present in the kidney [9].

Esculetin (Esc) is a coumarin derivative contained in many plants, such as Artemisia capillaris (Compositae), the leaves of Citrus limonia (Rutaceae), and Ceratostigma willmottianum [10,11] that are used as folk medicines. Various studies have reported Esc to possess antioxidant, antitumor, neuroprotective, and anti-inflammatory activities [12]. Esc has been reported to inhibit NMDA neurotoxicity by modulating the expression of NMDA [13]. The renoprotective role of NMDA antagonist has been suggested in ischemia-reperfusion-induced renal dysfunction [14] and drug-induced renal dysfunction [8]. Recently, the renoprotective role of Esc has been evidenced in streptozotocin induced diabetic rats [15,16]. Therefore, Esc was selected to evaluate its efficacy in genta-induced nephrotoxicity and its effect as NMDAR antagonist.

D-serine was used as a NMDA agonist in this study. It is relevant endogenous NMDAR ligand which act as coagonist at “glycine site” of NR1 subunit [17]. Therefore, this study has been designed to evaluate the role Esc mediated renoprotection against genta-induced nephrotoxicity and possible involvement of NMDAR.

METHODS

Animals
Sprague Dawley rats weighing (200-250 g) of age 3-5 months (National institute of Pharmaceutical Education and Research, Mohali, Punjab,
were exposed to normal cycle of light and dark. The experimental protocol was approved by the Institutional Animal Ethics Committee and care of the animals were taken as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest Government of India (1201/9/08/CPCSEA/013).

**Drugs and chemicals**

Esc and D-serine were purchased from Sigma-Aldrich and Himedia Laboratories Pvt. Ltd., India. Genta was obtained from Nitin Life sciences Ltd., India. Eosin and haematoxylin were procured from S.D. Fine Chemicals Ltd., India. Dithiobis nitro benzoic acid, reduced glutathione (GSH), and nitro blue tetrazolium were obtained from Loba Chemie, India. All other reagents used in the study were of analytical grade.

**Induction of nephrotoxicity**

The nephrotoxicity in rats was induced by administering genta at a dose of 100 mg/kg; i.p. for 7 days [18,19]. Animals were placed in metabolic cages for urine collection. On 8th day, animals were anesthetized using diethyl ether. The blood samples were collected using retro-orbital puncture, and rats were sacrificed by cervical dislocation. The plasma isolated from blood was used for estimation of creatinine, blood urea nitrogen (BUN), uric acid, sodium and potassium level. Moreover, the creatinine, sodium, potassium, and protein content in urine were estimated. The kidneys were removed and washed with 1.17% potassium chloride (KCl) solution. A part of renal tissue was preserved in neutral buffered formalin for histological studies. A small portion was used for estimation of superoxide anion generation (SAG), and the rest of tissue was minced and homogenized (10% w/v) in 1.17% KCl solution using teflon homogenizer. The contents were centrifuged at 800 g for 20 minutes. The pellet obtained was used for estimation of myeloperoxidase (MPO) activity, whereas the clear supernatant was used to estimate lipid peroxides and reduced GSH levels.

**Estimation of creatinine clearance**

The estimation of creatinine in plasma and urine samples was done by using commercially available kit by ERBA Diagnostics Pvt. Ltd., India.

**Estimation of BUN level**

The BUN level was estimated in plasma by using commercially available kit by Meril Diagnostics Pvt. Ltd., India.

**Estimation of plasma uric acid level**

The uric acid was estimated in the plasma sample using commercially available kit by ADI Diagnostics Pvt. Ltd., India.

**Estimation of fractional excretion of sodium**

The sodium level was estimated in the plasma and urine samples by using commercially available kit by Reckon Diagnostics Pvt. Ltd., India.

**Estimation of fractional excretion of potassium**

The potassium level was estimated in the plasma and urine samples by using commercially available kit by Reckon Diagnostics Pvt. Ltd., India.

**Estimation of microproteinuria**

The microproteins were estimated in urine samples by using commercially available kit by ERBA Diagnostics Pvt. Ltd., India.

**Estimation of MPO activity**

The MPO activity which is measured as an index of neutrophil accumulation was measured using method of Bradley et al. [20].

**Estimation of thiobarbituric acid reactive substance**

The quantitative measurement of thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation in renal tissue was performed according to method of Ohkawa et al. [21].

**Estimation of creatinine clearance**

The total protein content was determined using Lowry's method [22] using bovine serum albumin as a standard.

**Estimation of SAG**

The sag in the renal tissue has been evaluated using method described by Wang et al. [23].

**Hematoxylin and eosin staining**

The renal tissues preserved in 10% formalin were dehydrated in graded concentrations of ethanol, immersed in xylene and then embedded in paraffin. The sections of 4 μm thickness were cut and placed on slide using commercial Baker's mounting fluid. Paraffin wax was removed by warming the slide gently, until the wax melted and then was washed with xylene. This was followed by washings with absolute alcohol and water to hydrate the sections and stained with haematoxylin and eosin described by Clayden [24]. The hydrated sections were stained with hematoxylin for 15 minutes. The stained sections were washed with water and treated with 1% acid alcohol mixture for 20 seconds. The acid alcohol mixture was washed off with water, and sections were counterstained with 1% aqueous solution of eosin for 2 minutes. After washing with water to remove excess of eosin, the sections were dehydrated using absolute alcohol and then mounted using Canada balsam as mounting agent. The slides were observed for gross histopathological changes and neutrophil accumulation.

**Experimental protocol**

About 6 groups were employed in the present study, each comprising 6 rats.

- **Group I (control)**
  - Animals were exposed to normal conditions for 7 days.

- **Group II (Esc per se)**
  - Animals in this group were administered Esc (10 mg/kg; i.p.) for 7 days.

- **Group III (genta)**
  - Animals in this group were treated with genta (100 mg/kg; i.p.) for 7 days.

- **Group IV (genta + Esc 5 mg/kg)**
  - Animals were treated with Esc (5 mg/kg; i.p.) 1 hr before the administration of genta (100 mg/kg; i.p.) for 7 days.

- **Group V (genta + Esc 10 mg/kg)**
  - Animals were treated with Esc (10 mg/kg; i.p.) 1 hr before the genta (100 mg/kg; i.p.) administration for 7 days.

- **Group VI (D-serine + genta + Esc 10 mg/kg)**
  - D-serine (80 mg/kg; i.p.) was administered to animals for 7 days followed by the similar treatment as mentioned in Group V.

**Statistical analysis**

Results were expressed as mean ± standard error of mean. The data obtained from various groups was statistically analysed using one-way ANOVA followed by Tukey's multiple range test. The p<0.05 was considered to be statistically significant.

**RESULTS**

Effect of various pharmacological interventions on creatinine clearance (CrCl)

The administration of genta (100 mg/kg; i.p. for 7 days) produces significant decrease in CrCl, when compared to control rats. The administration of Esc per se (10 mg/kg; i.p. for 7 days) did not show any significant effect on CrCl. The administration of Esc (5 mg/kg; i.p.; 10 mg/kg; i.p.) for 7 days to genta-treated rats; attenuated genta
decreased level of CrCl. The administration of D-serine (80 mg/kg i.p.) + Esc (10 mg/kg; i.p.) + genta (100 mg/kg i.p.) for 7 days did not produce significant effect on CrCl (Fig. 2).

Effect of various pharmacological interventions on BUN level
The administration of genta (100 mg/kg; i.p. for 7 days) produces significant increase in BUN level, when compared to control rats. The administration of Esc per se (10 mg/kg; i.p. for 7 days) did not show any significant effect on BUN level. The administration of Esc (5 mg/kg; i.p.; 10 mg/kg; i.p.) for 7 days to genta-treated rats; attenuated genta increased level of BUN. The administration of D-serine (80 mg/kg; i.p.) + Esc (10 mg/kg; i.p.) + genta (100 mg/kg; i.p.) for 7 days did not produce significant effect on BUN level (Fig. 3).

Effect of various pharmacological interventions on plasma uric acid level
The administration of genta (100 mg/kg; i.p. for 7 days) produces significant increase in plasma uric acid level, when compared to control rats. The administration of Esc per se (10 mg/kg; i.p. for 7 days) did not show any significant effect on plasma uric acid level. The administration of Esc (5 mg/kg; i.p.; 10 mg/kg; i.p.) for 7 days to genta-treated rats; attenuated genta increased level of plasma uric acid. The administration of D-serine (80 mg/kg i.p.) + Esc (10 mg/kg; i.p.) + genta (100 mg/kg i.p.) for 7 days did not produce significant effect on plasma uric acid level (Fig. 4).

Fig. 1: Histopathological investigation, (a) normal control: Glomerular capillaries (black arrow) with both PCT (green arrow) and DCT (blue arrow) appears normal, (b) gentamicin treated group. Intestinal oedema seen by separation of tubules as well as congestion of capillaries. Tubules show epithelial degeneration, severe necrosis (red) and glomerular capillaries are widened (black), (c) esculetin (5 mg/kg) treated group: The histological features are improved as compared to gentamicin treated group. Glomerular capillaries retain their normal appearance and tubular epithelium is diminished, (d) esculetin (10 mg/kg/day) treated group: The histological features greatly improved and come to their normal structure of glomerular capillaries and tubular epithelium, (e) D-serine (80 mg/kg) + gentamicin (100 mg/kg) + esculetin (10 mg/kg) treated group: The histopathological feature witnessed tubular necrosis (black arrow), interstitial edema and cluster of inflammatory cells (neutrophil accumulation) (red arrow)

Fig. 2: Effect of various pharmacological interventions on creatinine clearance. Esculin (LD): Esculetin low dose (5 mg/kg, i.p.), Esculin (HD): Esculetin high dose (10 mg/kg, i.p.), values are expressed as mean ± standard error of mean (STDEV), n=6, one-Way ANOVA followed by Tukey’s multiple range test. *p<0.005 as compared to control. **p<0.005 as compared to gentamicin-treated group. ***p<0.005 as compared to esculetin high-dose treated group

Fig. 3: Effect of various pharmacological interventions on blood urea nitrogen level. Esculin (LD): Esculetin low dose (5 mg/kg, i.p.). Esculin (HD): Esculetin high dose (10 mg/kg, i.p.), values are expressed as mean ± standard error of mean (STDEV), n=6, one-Way ANOVA followed by Tukey’s multiple range test. *p<0.005 as compared to control. **p<0.005 as compared to Gentamicin-treated group. ***p<0.005 as compared to esculetin high-dose treated group

Fig. 4: Effect of various pharmacological interventions on plasma uric acid level. Esculin (LD): Esculetin low dose (5 mg/kg, i.p.), Esculin (HD): Esculetin high dose (10 mg/kg, i.p.), values are expressed as mean ± standard error of mean (STDEV), n=6, one-Way ANOVA followed by Tukey’s multiple range test. *p<0.005 as compared to control. **p<0.005 as compared to Gentamicin-treated group. ***p<0.005 as compared to esculetin high-dose treated group
Effect of various pharmacological interventions on microproteinuria

The administration of genta (100 mg/kg; i.p. for 7 days) produces significant increase in microproteinuria level, when compared to control rats. The administration of Esc per se (10 mg/kg; i.p. for 7 days) did not show any significant effect on microproteinuria level. The administration of Esc (5 mg/kg; i.p.; 10 mg/kg; i.p.) for 7 days to genta-treated rats; attenuated genta increased level of microproteinuria. The administration of D-serine (80 mg/kg i.p.) + Esc (10 mg/kg; i.p.) + genta (100 mg/kg i.p.) for 7 days did not produce significant effect on microproteinuria level (Fig. 5).

Effect of various pharmacological interventions on Fe K

The administration of genta (100 mg/kg; i.p. for 7 days) produces significant increase in Fe K level, when compared to control rats. The administration of Esc per se (10 mg/kg; i.p. for 7 days) did not show any significant effect on Fe K level. The administration of Esc (5 mg/kg; i.p.; 10 mg/kg; i.p.) for 7 days to genta-treated rats; attenuated genta increased level of Fe K. The administration of D-serine (80 mg/kg i.p.) + Esc (10 mg/kg; i.p.) + genta (100 mg/kg i.p.) for 7 days did not produce significant effect on Fe K level (Fig. 6).

Effect of various pharmacological interventions on Fe Na

The administration of genta (100 mg/kg; i.p. for 7 days) produces significant increase in Fe Na level, when compared to control rats. The administration of Esc per se (10 mg/kg; i.p. for 7 days) did not show any significant effect on Fe Na level. The administration of Esc (5 mg/kg; i.p.; 10 mg/kg; i.p.) for 7 days to genta-treated rats; attenuated genta increased level of Fe Na. The administration of D-serine (80 mg/kg i.p.) + Esc (10 mg/kg; i.p.) + genta (100 mg/kg i.p.) for 7 days did not produce significant effect on Fe Na level (Fig. 7).

Effect of various pharmacological interventions on TBARS

The administration of genta (100 mg/kg; i.p. for 7 days) produces significant increase in TBARS level, when compared to control rats. The administration of Esc per se (10 mg/kg; i.p. for 7 days) did not show any significant effect on TBARS level. The administration of Esc (5 mg/kg; i.p.; 10 mg/kg; i.p.) for 7 days to genta-treated rats; attenuated genta increased level of TBARS. The administration of D-serine (80 mg/kg i.p.) + Esc (10 mg/kg; i.p.) + genta (100 mg/kg i.p.) for 7 days did not produce significant effect on TBARS level (Fig. 8).

Effect of various pharmacological interventions on GSH level

The administration of genta (100 mg/kg; i.p. for 7 days) produces significant decrease in GSH level, when compared to control rats. The administration of Esc per se (10 mg/kg; i.p. for 7 days) did not show any significant effect on GSH level. The administration of Esc (5 mg/kg; i.p.; 10 mg/kg; i.p.) for 7 days to genta-treated rats; attenuated genta decreased level of GSH level. The administration of D-serine (80 mg/kg i.p.) + Esc (10 mg/kg; i.p.) + genta (100 mg/kg i.p.) for 7 days did not produce significant effect on GSH level (Fig. 9).

Effect of various pharmacological interventions on MPO activity

The administration of genta (100 mg/kg; i.p. for 7 days) and D-serine (80 mg/kg; i.p. for 7 days) produces significant increase in MPO activity.
administration of Esc per se (10 mg/kg; i.p. for 7 days) did not show any significant effect on SAG level. The administration of Esculin low dose (5 mg/kg; i.p.), Esculin (HD): Esculetin high dose (10 mg/kg, i.p.), values are expressed as mean ± standard error of mean (STDEV), n=6, one-Way ANOVA followed by Tukey’s multiple range test. *p<0.005 as compared to control. **p<0.005 as compared to Gentamicin-treated group. ***p<0.005 as compared to esculetin high-dose treated group

**DISCUSSION**

This study is based on ameliorative role of Esc-mediated renoprotection against genta-induced nephrotoxicity and possible involvement of NMDAR. Genta is an antibiotic that exhibits a broad spectrum of activity and is particularly valuable in severe sepsis. Its use is, however, restricted because of the development of ototoxicity and nephrotoxicity [25,26]. Nephrotoxicity has been related to a selective accumulation of genta in the renal cortex and resulting morphologic lesions of proximal tubules [26,27]. Genta is associated with an induction of tubular necrosis, epithelial oedema of proximal tubules, cellular desquamation,
The role of Esc in genta-induced nephrotoxicity and involvement of NMDAR was explored for the first time in this study. The administration of genta (100 mg/kg) for 7 days resulted in significant renal damage as indicated by decreased CrCl and increased levels of BUN, uric acid, Fe\textsubscript{2+} microproteinuria and significant rise in oxidative stress parameters TBARS, SAG and MPO along with depletion of GSH, an established indicator of antioxidant defense of the body. The treatment with Esc (5 mg/kg; 10 mg/kg) significantly attenuated genta-induced renal damage.

Hence, on the basis of above discussion, it is concluded that the treatment with Esc protects kidneys from genta-induced oxidative stress and dysfunction. Moreover, the administration of D-serine abolished the effect of Esc and thus indicating that NMDAR may be involved in the genta-induced nephrotoxicity.

REFERENCES

21. Okhawa H, Oku N, Yagi K. Assay for free peroxides in animal tissues

327