

Nephroprotection of *Withania somnifera* root extract against gentamicin induced nephrotoxicity: a histological evaluation in experimental Wistar rats

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ABSTRACT

Background: Gentamicin, an aminoglycoside group of drug, used against aerobic gram negative bacteria, is known for nephrotoxicity. Herbal products have a special place in the world of pharmaceuticals with their safety, efficacy and cost effectiveness. *Withania somnifera* (Ashwagandha) roots had known since long for its antioxidant status and free radical scavenging property. So *W. somnifera* can be used as nephroprotective agent because of free radical scavenging property.

Methods: Total 54 rats were randomised in 3 groups named G10, G20 and G30 according to 10, 20 and 30 days of treatment. In each groups, rats were randomly assigned to any of the three subgroups i.e., control C group [received normal saline (2 ml/100 gm/day) orally consecutively for test duration], gentamicin treated GT group [received normal saline (2 ml/100 gm/day) orally consecutively for test duration and intraperitoneal gentamicin (40 mg/kg) once daily for last five days] and *W. somnifera* treated WST group [received *W. somnifera* orally (500 mg/kg/day) for the test duration and intraperitoneal gentamicin (40 mg/kg) once daily for last five days]. Rats were sacrificed 24 hours after the last dose of gentamicin injection. Excised kidneys were weighted and prepared for histological examination.

Results: The mean weight of kidneys in GT group was significantly higher than the WST group in all test durations indicating the antioxidant and free radical scavenging property. This was also reflected histologically as WST group kidney showed less amount inflammation as compared to GT group.

Conclusions: *W. somnifera* root extract provide nephroprotection against gentamicin induced nephrotoxicity.

Keywords: Nephroprotection, Gentamicin, *Withania somnifera*, Ashwagandha, Tubular epithelium

INTRODUCTION

Aminoglycoside is a gram-negative antibacterial therapeutic agents that inhibit protein synthesis and contain an amino-modified glycoside sugar.¹ Aminoglycoside is useful primarily in infections involving aerobic gram-negative bacteria, such as *Pseudomonas*, *Acinetobacter* and *Enterobacter*. Aminoglycoside are one of the important classes of antimicrobial agents which are used for more than 50

years.² One important among them is gentamicin. Gentamicin has nephrotoxic potential. Gentamicin is well recognized to produce renal tubular necrosis mainly in the proximal tubule. Du et al found in their study that gentamicin enhanced superoxide anion and hydroxyl radical production in renal cortical mitochondria. They proposed a role of hydroxyl radicals in pathogenesis of gentamicin toxicity by inducing suppression of Na⁺K⁺ ATPase activity and DNA synthesis in proximal tubules.³ These processes results in accumulation of gentamicin in

renal proximal convoluted tubules in a concentration, 50-100 times greater than the serum. Thus serum half-life of aminoglycoside, which is few hours is prolonged (4-5 days in proximal tubule cells).⁴ This drug causes generation of reactive oxygen species, which induces cell injury and necrosis via lipid peroxidation. Administration of gentamicin leads to elevation in serum creatinine levels, urea levels, and renal tubular necrosis along with renal failure.^{5,6} Among several aminoglycoside antibiotics, the grade of nephrotoxicity caused has been reported to be in the following order as neomycin>gentamicin>tobramycin.⁷ Gentamicin nephrotoxicity occurs in about 15-30% of treated subjects.

Herbal products have a special place in the world of pharmaceuticals with their safety, efficacy and cost effectiveness. The World Health Organization found that 80% of the world's population depends on medicinal plants for their healthcare needs, and more than 30% of the pharmaceutical preparations are based on plants.⁸

Ashwagandha (*Withania somnifera*), also known as Indian winter cherry is an important ancient plant, the roots of which have been employed in Indian traditional systems of medicine, Ayurveda and Unani. In Sanskrit it is called Ashvagandha (asva 'horse', gandha 'smell') which means 'smell of the horse', because the root of the infected plant has the smell of the horse urine.⁹ *W. somnifera* is a small, erect, evergreen woody under shrub belonging to *Solanaceae* family. The plant is chemically very complex and more than 80 compounds have been derived from it. It has been used in various diseases such as rheumatism, leprosy and arthritis.¹⁰ Among the other parts of this plant, the root has been considered to be the most active part for therapeutic purposes. The constituents of *W. somnifera* roots are the steroidal alkaloids and steroidal lactones. They belong to a class of constituents called the withanolides with the main active chemical constituent being withaferin A, a phytosteroid.¹¹ Much of pharmacological activity in this plant has been attributed to two main withanolides i.e., withaferin A and withanolide D.¹² Previously, Prakash et al stated that *W. somnifera* extract, for its antioxidant status and free radical scavenging property on 7, 12-dimethylbenz (a) anthracene, induced skin cancer in swiss albino mice.¹³

Ashwagandha can grow easily everywhere in India and its roots are known for antioxidant property since long, it can be used as nephroprotective drug against gentamicin induced nephrotoxicity. The present study was done to evaluate the nephroprotective action of the Ashwagandha root extract.

Phytomorphology of *Withania somnifera*

W. somnifera is a small or medium under shrub, 30-150 cm height, erect, greyish, branching perennial, with strong disagreeable odour like horse's urine coming from tuberous roots. The leaves are in an alternate manner (opposite on flowering shoots), normally broadly ovate. Flowers are generally small, greenish. Seeds are normally

yellow. *Withania somnifera* can be identified by the red berry like fruit of about 6 mm size enclosed by the brownish, papery, turgid calyx.⁹

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METHODS

This study was conducted in the Department of Pharmacology, L. L. R. M. Medical College, Meerut, UP, India from January 2015 to October 2015. Approval was taken from Institutional animal ethical committee. (Approval no. IAEC/2015/2 dated 14/5/15 of Lala Lajpat Rai Memorial Medical College, Meerut, India, registered under CPCSEA India (Registration No. 819/04/ac/CPCSEA). Total duration of study was ten months.

Animal maintenance

The certified pathogen-free healthy Albino Wistar rats of either sex, weighing about 180 gm, were obtained from the central animal house of the institute. The selected rats were kept under controlled temperature (25⁰ C) and alternating periods of light and darkness of 12 hours each. The rats had free access to standard rat pellet diet (Vet care India Ltd.) and tap water ad libitum. After seven days of acclimatization, rats were considered fit for study.

Plant material

W. somnifera (Ashwagandha) dry roots were procured from the Ayurveda medicinal shop in Meerut, Uttar Pradesh, India. The dry roots were identified and authenticated by the scientists in Botany Department of Chaudhary Charan Singh University, Meerut, (UP) India.

Preparation of the root extract of *W. somnifera*

The plant's root was dried for two days at 40⁰ C, crushed and powder was separated. Total 50 gram powder was mixed in 250 ml of distilled water for 18 hours in a soxhlet apparatus. Extract of the root of *W. somnifera* in distilled water was collected. The extract was dried and stored at 0-4⁰ C. When needed the extract was suspended in water and used in the study. Aqueous extract of *W. somnifera* roots as a test drug was used.

Estimation of LD50

LD50 of *W. somnifera* root extract was determined on wistar rats by standard procedure. The acute oral toxicity study and estimation of LD50 was done according to the OECD guideline 423.¹⁴

Gentamicin

Commercially available injectable preparation (Genticyn manufactured by Abbott Healthcare Pvt. Ltd.) was used to induce gentamicin nephrotoxicity.

Experimental design

Nephroprotection was evaluated for three periods of 10, 20, 30 days durations and groups were named G10, G20, and G30 respectively. Rats were randomized in three groups of 18 rats each. Rats of each main groups were randomized to any of the three subgroups i.e., control (C), gentamicin treated (GT), *W. somnifera* treated (WST) groups of six rats each.

Group C

This was the control group and was given normal saline (2 ml/100 gm/day) orally once a day, every day for test duration.

Group GT

In this group, normal saline (2 ml/100 gm/day) was given orally once a day consecutively for test duration and injection gentamicin (40 mg/kg) was injected intraperitoneally once daily for last five days.

Group WST

This group received *W. somnifera* orally (500 mg/kg/day) as a single dose in morning, before giving feed for the test duration and, injection gentamicin (40 mg/kg) was given intra-peritoneally once daily for last five days.

Rats were sacrificed 24 hours after the last dose of gentamicin injection (on 11th, 21st and 31st day).

The test compounds were administered by the oral gavage method. Rats were fasted three to four hours prior to and one hour after administration. On the last day of study, rats of all groups were kept on fasting for 24 hours (tap water remained freely available) after which they were sacrificed under ketamine (75 mg/kg) and xylazine (10 mg/kg) anaesthesia given intra-peritoneally.¹⁵

The kidneys were excised from the animal and washed with the ice-cold saline. Kidneys were wiped with tissue paper and weighted immediately by electric balance analyser. After this, kidney was sectioned longitudinally in two halves and fixed in 10% buffered neutral formalin, dehydrated and cleared with graded alcohol and xylene respectively. This was followed by embedding in paraffin wax from which blocks were prepared. Sections of 5 µm thickness were prepared from the blocks using a microtome. These were processed in alcohol xylene series and were stained with Harris haematoxylin and eosin stain and subjected to histopathological examination.¹⁶

Histopathological examination

It was done in Pathology department under light microscope. Various structure of kidney were evaluated like congestion of glomerulus and tubule, epithelial desquamation, blood vessels congestion, interstitial edema, inflammatory cells, tubular casts and areas of necrosis.

Histological changes in kidney seen under light microscope after gentamicin induced toxicity were graded according to system adopted by Houghton et al.^{17,18}

Grade 0: Normal (-).

Grade 1: Tubular damage involving <25% of cortical tubules (+).

Grade 2: Tubular damage involving 25% and <50% of cortical tubules (++)

Grade 3: Tubular damage involving 50% and <75% of cortical tubules (+++).

Grade 4: Tubular damage involving 75% of cortical tubules (++++).

Statistical analysis

Mean±SE was calculated for each group. Normality of the data was assessed using Kolmogorov-Smirnov test. Statistical analysis was done using t-test for normally distributed data and using Mann-Whitney test for non-normal data. P-values <0.05 were considered as significant. P-values were estimated by referring to appropriate tables.

RESULTS

Histological effects of gentamicin and *W. somnifera* treatment on kidney

Histological evaluation was done in the department of pathology and slides were examined under light microscope. Patchy tubular necrosis along with hyaline cast in the tubular lumina was the predominant morphological feature in GT group. Dropped out tubular cells were also seen in the lumina of some of the renal tubules. Epithelial cells had large vacuoles in it. Some cells had large prominent nuclei suggestive of regenerating cells. The regenerating cells were recognized by enlarged nuclei. Glomeruli were least affected part in kidney. The glomeruli showed mild hyper-cellularity and congestion in most of the sections. There were infiltration of polymorphonuclear leukocytes, lymphocytes, plasma cells and macrophages in renal interstitium particularly around the necrotic tubules. The renal vascular changes were limited to focal area of congestion.

There were very less damage to tubular epithelium and infiltrations of inflammatory cells less as compared to GT group after 10, 20 and 30 days of pre-treatment. Areas of necrosis were few in number and vascular congestion was

also less as compared to GT group. Regenerating cells were densely populated in the slides of 20 and 30 days group. The histological changes were graded as in below mentioned table (Table 1).

Table 1: Histopathological grading of kidney under light microscope at 10, 20 and 30 day duration.

Histopathological features	Histological grading at 10 days			Histological grading at 20 days			Histological grading at 30 days		
	C	GT	WST	C	GT	WST	C	GT	WST
Glomerular congestion	0	3	1	0	2	0	0	1	0
Peritubular congestion	0	2	1	0	1	1	0	2	1
Epithelial desquamation	0	2	2	0	1	1	0	1	1
Blood vessel congestion	0	2	1	0	2	1	0	2	1
Interstitial oedema	0	3	2	0	2	0	0	2	0
Inflammatory cells	0	3	1	0	2	1	0	1	0
Tubular casts	0	2	2	0	1	1	0	1	0
Necrosis	0	1	0	0	1	0	0	1	0

Kidney weights (in grams)

Mean kidney weight of GT group was significantly high than the C group in all test durations. Mean kidney

weight of WST group was significantly high as compared to GT group in all test duration but only at 10 days duration as compared to C group. No significant difference was found between WST and C group at 20 and 30 days duration (Table 2).

Table 2: Weight of kidneys in C, GT and WST groups at different test durations (n=6).

Duration of treatment (days)	Kidney weight in C group (Mean±SE) (in gram)	Kidney weight in GT group (Mean±SE) (in gram)	Kidney weight in WST group (Mean±SE) (in gram)
10	0.58±0.04	1.03±0.10 ^a	0.78±0.04 ^{cλ}
20	0.59±0.03	1.0±0.12 ^β	0.63±0.04 ^{εμ}
30	0.56±0.04	1.01±0.09 ^γ	0.58±0.02 ^{ην}

^ap<0.05as compared to 10 days of control, ^βp<0.05as compared to 20 days of control, ^γp<0.05as compared to 30 days of control, ^εp<0.05as compared to 10 days of control, ^εp>0.05 as compared to 20 days of control, ^ηp>0.05 as compared to 30 days of control, ^λp<0.05as compared to 10 days of GT, ^μp<0.05as compared to 20 days of GT and ^νp<0.05as compared to 30 days of GT.

DISCUSSION

Drugs are a major contributor to acute renal failure. Renal toxicity attributable to aminoglycoside is of major clinical concern because of their widespread use.¹⁹⁻²¹ The number of patients developing nephrotoxicity increases with duration of therapy reaching 50% if the therapy is continued for 14 days or more.²² Renal damage caused by gentamicin depends upon the dose and duration of the treatment. Different researchers used different doses, ranging between 8 and 80 mg/kg/day to produce renal damage.²³ However, these toxic effects are only associated if the drug is taken five to ten times normal doses.^{24,25}

So protection from aminoglycoside induced nephrotoxicity by any means would be of great clinical importance. Gentamicin induced nephrotoxicity model is one of the important and validated models used to study nephroprotective potential of drugs and phytopharmaceuticals. A direct tubular necrosis is also observed during nephrotoxicity.

After 10 days of study

The mean kidney weights were significantly high in GT group as compared to C group (p<0.05). Gentamicin induced nephrotoxicity causes edema and inflammatory changes in proximal tubular epithelial cells thereby increase the kidney weight.²⁶ This was also correlated histologically as slides showed intense inflammatory cells in the interstitium and distorted architect of proximal tubular epithelium with marked vasodilatation (Figure 1).

Mean weight of kidney of WST group was significantly high as compared to C group but significantly low as compared to GT group (p<0.05). This was also correlated histologically as necrosis was very few and hyaline cast in the tubule were infrequent. Inflammatory cells were also less as compared to GT group (Figure 2). *W. somnifera* roots extract contains withanolides which inhibits cyclooxygenase enzyme, lipid peroxidation and proliferation of tumor cells. Thus it inhibits inflammation by inactivating nuclear factor kappa-B activation. This property is responsible for limiting the inflammation activity to minimal level.²⁷

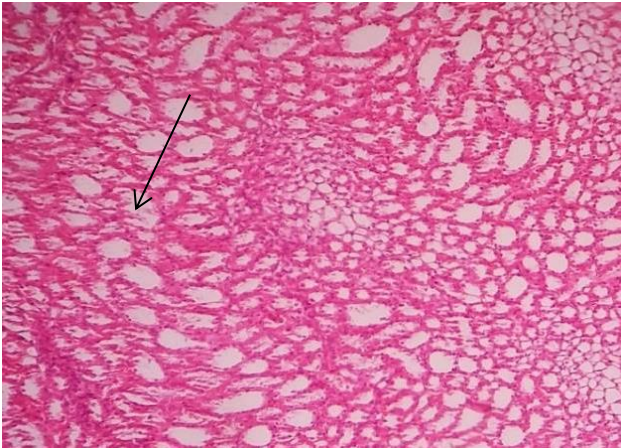


Figure 1: Histology of GT group at 10 days duration showing distorted tubular epithelium (black arrow) and cellular edema.

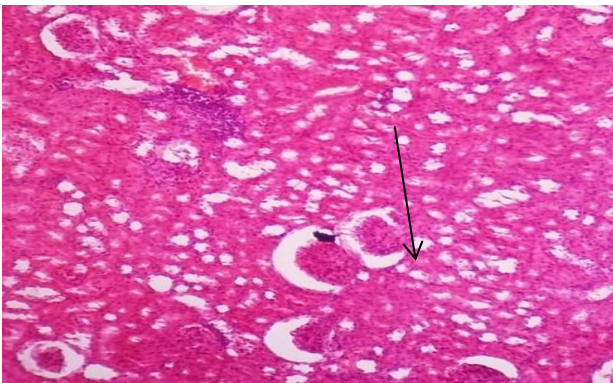


Figure 2: Histology of WST group at 10 days duration showing minimal distortion of tubular epithelium (black arrow) and cellular edema.

After 20 days of study

In 20 days duration study kidney weight in GT group was significantly high as compared to C group ($p < 0.05$). This was again due to intense inflammatory injury of kidney caused by gentamicin. Reactive oxygen species and reactive nitrogen species generated by gentamicin, plays crucial role in the initiation, and progression of nephrotoxicity.²⁸

The mean weight of kidney in WST group was not significantly different to C group ($p > 0.05$) but significantly low as compared to GT group ($p < 0.05$). This means *W. somnifera* as a nephroprotective drug, take some days to work on kidney because the inflammation at 10 days was not comparable to control group. This was also correlated histologically as tubular architect were less distorted and regenerating cells were numerous in number as compared with 10 days slide of WST group (Figure 3). On looking these slides under 10X and 40X magnifications, regenerating cells were recognized by enlarged nuclei and smooth border. Glomerular

congestion and interstitial edema was absent though it was present at 10 day duration.

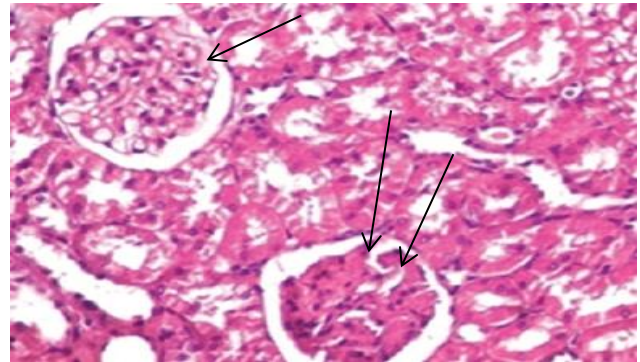


Figure 3: Histology of WST group at 20 days duration showing inflammatory cells (single black arrow) and near normal tubular epithelium (two black arrow).

After 30 days of study

As expected the mean kidney weight was significantly high in GT group as compared to C group because gentamicin was injected in last 5 days only in all test durations. Glomeruli were almost normal in majority of slides. Gentamicin induced nephrotoxicity is characterized by direct tubular necrosis, without morphological changes in glomerular structures.²⁹

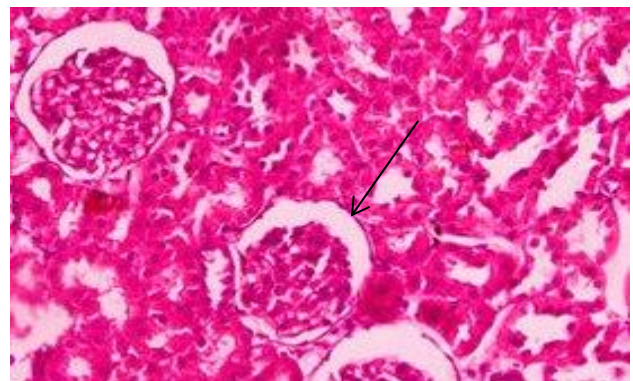


Figure 4: Histology of WST group at 30 days duration showing normal tubular epithelium (black arrow).

Mean weight of kidney in WST group was not different to C group but significantly low as compared to GT group ($p < 0.05$). Histologically, there was reorganisation of tubular epithelium and inflammatory cells were absent. Hyaline cast were few in number and vascular congestion was also less as compared to 20 days duration slide of WST treated kidney (Figure 4).

Gentamicin toxicity causes permanent destruction of some of the tubule that reflect as fibrosis and scarring of sub-capsular as well as deeper layer in the kidney. This is the cause of suboptimal kidney function even after near normal structural rebuilt of kidney.¹⁸

CONCLUSION

This study strengthens the fact that Ashwagandha root extract had antioxidant and free radical scavenging property. This extract can be used as nephroprotection against gentamicin induced nephrotoxicity. We need more study with increase duration of gentamicin injection to see its action on kidney.

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Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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