Protective Effects of Rutin and Naringin on Gentamycin Induced Testicular Oxidative Stress

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ABSTRACT

***Aim**: The aim of this study was to investigate the protective effects of bioflavonoid compounds i.e. rutin and naringin on gentamycin induced testicular oxidative stress in rats.*

***Method**: A total of 42 male wistar albino rats were divided in to 7 groups, each contains 6 rats. Gentamycin, 5 mg/kg, I.P was administered to the rats from day 1 to day 10. Rutin and naringin were given in different doses (5mg/kg, 10/kg) for a period of 35 days from day 1 to day 35 to the gentamycin treated animals. A sham control group animals with water, control group animals with gentamycin and vehicle control group animals with 0.1% sod CMC were also maintained until completion of 35 days. On 36th day bilateral orchiectomies were performed for all the animals. The testicular tissue was evaluated for various sperm parameters, biochemical estimations and histological changes.*

***Result**: Animals treated with 5mg/kg and 10mg/kg of rutin and naringin (Groups 4, 5, 6, 7) have shown significant and dose dependent reduction in MDA levels and increase in levels of antioxidant enzymes, SOD and Catalase when compared to control group animals (Group 2). Sperm count, motility, viability were also protected and normalized with rutin and naringin. Specimens from group 2 had a histological injury with disordered germinal cells. Drug treated groups shown improved testicular architecture. Rutin was more effective in all the parameters when compared to Naringin. Both the bioflavonoids were effective in reducing the gentamycin induced testicular oxidative stress.*

***Conclusion**: Rutin and Naringin pretreatment have shown protective and beneficial effect on gentamycin induced oxidative stress in rats.*

***Key words**: Bioflavonoids, naringin, gentamycin,testicular oxidative stress.*

Gentamisinin Meydana Getirdiği Testiküler Oksidatif Streste Rutin ve Naringinin Koruyucu Etkisi
Amaç: *Bu çalışmanın amacı ratlarda gentamisinin neden olduğu testiküler oksidatif stress üzerine bioflavonoid bileşiklerinden olan rutin ve naringinin koruyucu etkisini araştırmaktır.*
Metod: *Toplam 42 adet wistar albino ratlar 6'lı gruplar halinde 7 gruba ayrıldı. Gentamisin 5 mg/kg 1. günden 10. güne kadar intraperitoneal olarak uygulandı. Rutin ve naringin 5 mg/kg ve 10 mg/kg dozlarında gentamisin ile birlikte verilmek üzere toplam 35 gün verildi. Kontrol grubu olarak 35 gün boyunca gentamisin, 0.1% sod CMC ve su alan ratlar çalışmada kullanıldı. Çalışmanın 36. günü tüm ratlara orşiektomi yapılarak sperm parametreleri, histolojik değişiklikler ve biokimyasal parametreler değerlendirildi.*
Bulgular: *5 mg/kg ve 10 mg/kg rutin ve naringin ile tedavi edilen ratlarda (grup 4,5,6,7) kontrol grubuna göre doz bağımlı olarak anlamlı ölçüde serum MDA seviyesinde azalma ve SOD ve katalaz gibi antioksidan enzim düzeylerinde artış tespit edildi. Rutin ve nargirin tedavisi altında sperm sayısı, motilitesi ve viabilitesi korunmuş olarak bulundu. Grup 2'den elde edilen hücre örneklerinde histolojik olarak germinal hücre hasarı mevcuttu. İlaçla tedavi edilen gruplarda testiküler dokuda iyileşme saptandı. Naringin ile karşılaştırıldında rutin tüm parameterler üzerine daha iyi etkilere sahipti. Her iki bioflavonoid gentamisin nedenli testiküler stresste etkili bulundu.*
Sonuç: *Rutin ve naringin tedavisi ratlarda gentamisin nedenli oksidatif stressi zaltmada koruyucu ve etkin bir tedavi sağlamıştır.*
Anahtar kelimeler: *Bioflavonoid, naringin, gentamycin, testiküler oksidatif stres*

INTRODUCTION

Infertility is the growing problem worldwide and estimated to be about 13 to 18% (1). Infertility is defined as the state in which a couple wanting a child cannot conceive after 12 months of unprotected intercourse (2). Among the causes of infertility, about half of them could be traced to the male partner (3). Increasing evidence has established a link between chemical/drug exposure and generation of reactive oxygen species (ROS) (4). Oxidative stress is one of the major causative factors for various diseases and organ toxicities. Testicular toxicity due to increased oxidative stress impairs fertility (5). Antibiotics are commonly prescribed for a variety of diseases. Gentamycin is an aminoglycocide antibiotic used in neonatal sepsis and other systemic infections caused by gram negative microorganisms. Gentamycin possesses few important toxic effects including ototoxicity and nephrotoxicicy. Now there is sufficient evidence to claim, both ototoxicity and nephrotoxicicy are due to the generation of reactive oxygen species (5-7).

Gentamycin inhibits cell division of gem cells and protein synthesis in the testis, induction of cell death in the seminal vesicle. Gentamycin was known to reduce sperm count, sperm motility, and sperm viability. Gentamycin induces these changes by increasing the free radical formation and lipid peroxidation, and by decreasing antioxidant enzyme levels (5). Bioflavonoids are classical examples for antioxidants. They are proved as beneficial agents for free radical scavenging. Rutin and Naringin belong to the class of bioflavonoids which are proved as potent antioxidants. Extensive studies were conducted on both these drugs. They were proved in our laboratory as well as by the other authors as protective agents in oxidative stress of cardiac ischemia-reperfusion injury and cerebral ischemia-reperfusion injury (8-17).

However to the best our knowledge, there were no reports describing their protective role against Gentamycin induced testicular oxidative damage. The present study was conducted to evaluate the protective role of bioflavonoids rutin and naringin against gentamycin induced testicular oxidative damage in vivo. The extent of testicular damage caused by gentamycin induced oxidative stress and protective effects of rutin and naringin were evaluated by measuring 1) the activities of biochemical parameters like MDA, SOD and Catalase 2) the sperm count, sperm motility, Sperm viability. In addition histological studies were done to assess the structural changes in testes.

MATERIALS AND METHODS

Animals

Male albino wistar rats (National Institute of Nutrition, Hyderabad, India) weighing 165 – 210 g were used in the study. They were maintained under standard laboratory conditions at 25 ¤ 20 C, relative humidity 50 ¤ 15% and normal photoperiod (12 h dark/ 12 h light) and were used for the experiment. Commercial pellet diet (Rayons biotechnologies Pvt Ltd, India) and water were provided ad libitum. The experimental protocol has been approved by the Institutional Animal Ethics Committee and by the Animal Regulatory Body of the Government (Regd. No. 516/01/A/CPCSEA).

***Table1**. Effects of rutin and naringin on biochemical and sperm parameters*

Groups	MDA	SOD	Catalase	Sperm count (millions/ml)	Sperm motility (%)	Sperm viability (%)
1	207.89±14.07	1649.46±6.94	25.67±2.77	30.5±3.50	56.16±2.78	73.16±2.31
2	334.99±5.84	1084.62±18.15	12.66±0.86	19.5±1.87	24.16±2.48	35.83±1.32
3	328.45±13.31	1108.01±11.36	11.15±0.70	20.5±2.16	24.66±2.16	33.66±1.86
4	252.83±11.31	1459.04±8.65	17.43±1.51	26.33±1.50	44.5±2.58	64.16±2.99
5	220.88±9.01	1622.85±6.37	24.35±1.44	29.16±2.48	50.66±4.45	70.33±4.76
6	271.07±5.40	1363.87±6.19	15.71±0.72	24.16±2.48	43.66±3.50	60.16±2.22
7	252.22±4.87	1577.89±7.46	21.38±2.55	28±2.60	52.33±2.73	66.66±7.58

Chemicals

Gentamycin was purchased from Biochem Pharmaceuticals Ltd, Mumbai, INDIA. Rutin and naringin were procured from sigma chemicals, St Louis, USA. Thiopentone sodium was purchased from neon labs, Mumbai, INDIA. All other chemicals and reagents were used of analytical grade.

Experimental Groups

Total 42 animals were selected for the study and divided in to 7 groups each containing 6 rats. Group 1 is the sham control group, received only water. Group 2 is the control group and rats have received gentamycin 5mg/kg once daily for 10 days. The drug was dissolved in water for injection and given in I.P route. The dose and duration of treatment was similar as in humans. The route of administration was different from humans, but selected based on earlier reports, as I.P route is simple and accurate procedure (5). Group 3 animals act as vehicle control and have received both gentamycin 5mg/kg and 0.1% sodium carboxy methyl cellulose. Groups 4,5,6,7 were test groups which received both gentamycin and test drugs as described below: Group 4 received gentamycin and Rutin 5mg/kg. Group 5 received gentamycin and Rutin 10mg/kg. Group 6 received gentamycin and Naringin 5mg/kg. Group 7 received gentamycin and Naringin 10mg/kg. Both rutin and naringin were given daily for 35 days continuously.

Experimental protocol

After completion of 35 days of drug treatment, the animals were sacrificed with lethal ether anesthesia and laporatomy was conducted. Testes and epididymis were collected. The epididymis was used for the evaluation of sperm parameters. The right testis was processed for histopathological studies and left one was homogenated for biochemical estimations.

Biochemical Parameters Estimation

Malanoldehyde (MDA) levels in the testicular tissue were measured by the method developed by Ohkawa et al. (18). This is based on measurement of absorbance of thiobarbituric acid malanoldehyde. The tissue MDA levels were expressed as nmol/g tissue. Super oxide dismutase (SOD) activity was determined by the method developed by Fridovich (19). This method was based on inhibition of reaction of super oxide radicals with phenyl tetrazolium chloride. The specific activity was expressed in terms of units for mg of protein. Catalase activity was measured based on the method Aebi (20). Activity of catalase was based on the disappearance of hydrogen peroxide. Activity of catalase was expressed as ¤moles of H_2O_2 metabolized/mg protein/min. One unit was defined as 1pmol of H_2O_2 consumed per minute, and the specific activity was reported as units per milligram of protein. Protein was estimated by the method developed by Lowry (21).

Collection of spermatozoa for evaluation of sperm count, sperm motility, sperm viability

Epididymal spermatozoa were collected by cutting the cauda region of the epididymis in to small pieces in 2ml of normal saline pre warmed to 37°C. Sperm was forced out of the cauda epididymis with fine forceps by putting pressure on lower region of cauda epididymis, not forcing out excess material i.e. immature cells. In this study sperm motility, count, and viability were evaluated by using conventional methods (11,22,23). Progressive sperm motility was done immediately after collection of sperm. Number of motile spermatozoa were calculated per unit area and expressed as percentage sperm motility. Sperm counts were done using haemocytometer and results were expressed as millions/ml of suspension.

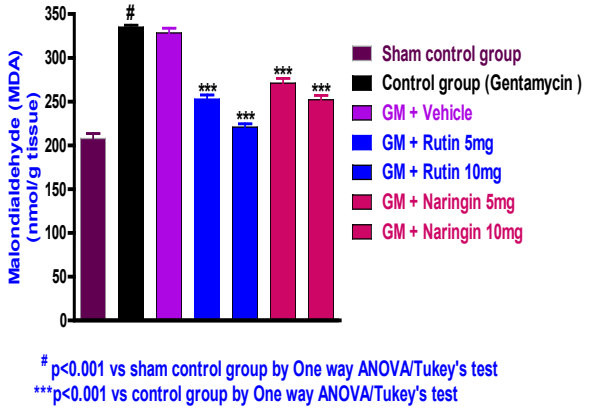

Figure 1. *MDA levels before and after treating with rutin and naringin.*

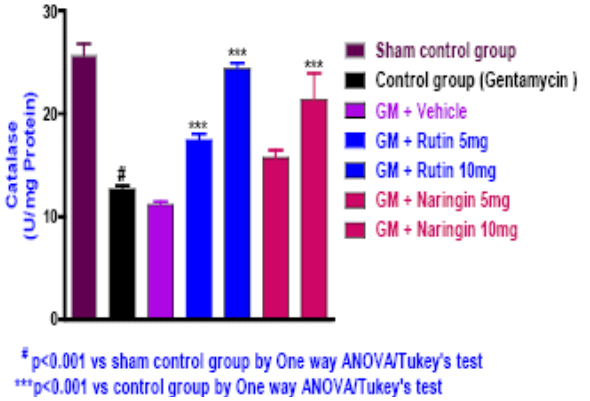

Figure 2. *Catalase levels before and after treating with rutin and naringin.*

Sperm viability was done using Eosin and Nigrosin stain. The dead sperm took up the stain. Hundred sperm cells were counted in order to obtain the percentage of live/death ratio.

Histopathological examination

The testis were fixed in 10% formalin and embedded in paraffin. Five-micron thick sections were prepared and stained with hematoxylin and eosin (H&E). The tissue sections were evaluated under light microscopy by a blinded pathologist.

Statistical analysis

The results are expressed as mean±SD. Differences in tissue lipid peroxide levels, SOD and CAT were determined by factorial one-way analysis of variance. Individual groups were compared using Tukey's test. Differences with $p<0.001$ were considered statistically significant. Statistical analysis was performed using Graph Pad Prism software (Version 5)

RESULTS

Effects on biochemical Parameters

MDA, SOD, Catalase levels in testicular tissue were given in Table 1. There was significant increase in MDA levels ($p<0.001$) and reduction of antioxidant enzymes

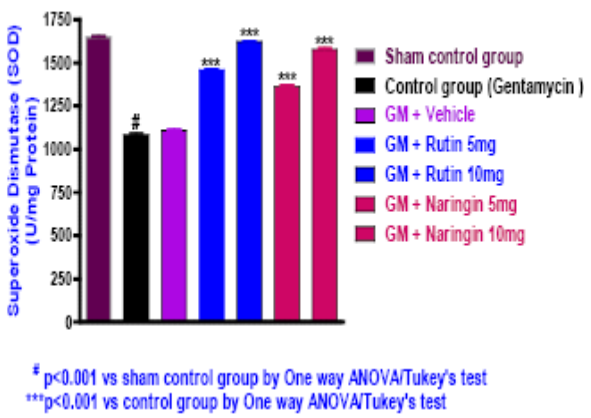

Figure 3. *SOD levels before and after treating with rutin and naringin.*

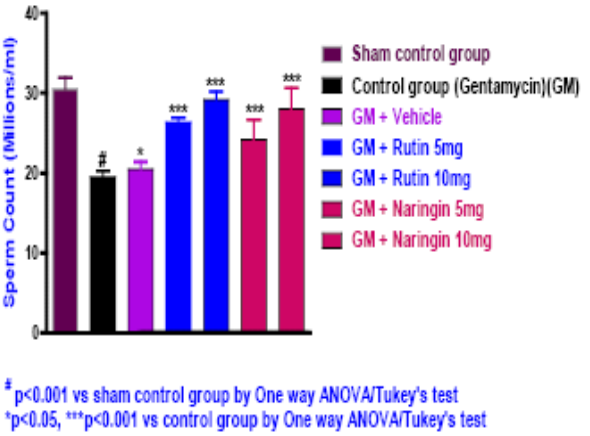

Figure 4. *Sperm count before and after treating with rutin and naringin.*

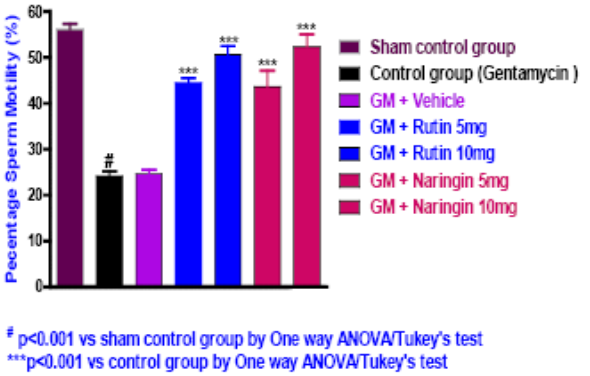

***Figure 5**. Sperm motility before and after treating with rutin and naringin.*

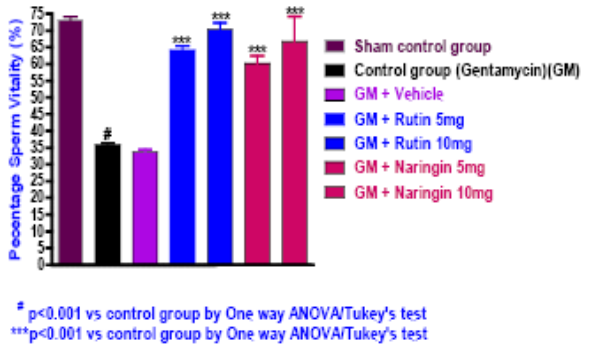

***Figure 6**. sperm viability before and after treating with rutin and naringin.*

SOD and catalase levels in gentamycin treated group when compared to sham control group. Rutin treatment (5mg & 10mg) has significantly ($p<0.001$) and dose dependently decreased the MDA levels and increased SOD and Catalase levels when compared to control group. Similarly, Naringin treatment (5mg & 10mg) also has significantly ($p<0.001$) and dose dependently decreased the MDA levels and increased SOD and Catalase levels when compared to control group treated with gentamycin. Rutin is slightly more efficacious than Naringin in reversing the degree of oxidative stress (Figures 1, 2 ,3).

Effects on Sperm parameters

Sperm count, motility and viability were significantly affected by gentamycin in control group. There was drastic fall in the sperm count and reduction in percentages of progressively motile and viable spermatozoa. Rutin treatment (5mg & 10mg) has significantly ($p<0.001$) and dose dependently increased sperm count, percentages of motile and viable Spermatozoa when compared to control group treated with gentamycin. Similarly, Naringin treatment (5mg & 10mg) also has significantly ($p<0.001$) and dose dependently increased percentage Sperm vitality, percentage of sperm motility and sperm count when compared to control group treated with gentamycin. Both Rutin and Naringin have shown almost similar degree of efficacy in the above sperm parameters (Figures 4,5,6).

Effects on histopathology of testicular tissue

Gentamycin treated control group animals showed significant degeneration of the seminiferous tubules with necrosis of spermatocytes, spermatids. Epithelial sloughing, epithelial gaps were observed.(Figure 8) Normal testicular architecture observed in figure 7 was disturbed in gentamycin treated group. Treatment with rutin and naringin showed structurally and functionally active seminiferous tubules similar to those of the sham control (Figures 9, 10). Particularly higher doses of rutin and naringin (10mg/kg) showed similar and maximum protective effect in histological examination.

DISCUSSION

In the last few years, there was a marked fall in the quality of semen produced. These changes in semen quality are reportedly due to the environmental factors like misuse of chemicals, various other pollutants and drugs. Drugs like antibiotics are used in variety of diseases right from the child hood. Gentamycin is an aminoglycocide antibiotic and used to treat infections of gram negative organisms (5). It is used in adults and children at the dose level of 3.0 to 7.5mg/kg for a period of 7 to 10 days. Its ADR profile includes ototoxicity and nephrotoxicicy, which are believed to occur due to severe oxidative stress caused by the gentamycin in its regular dosage (21).

There were few reports suggest about the role of gentamycin in reproductive system. It was found to inhibit cell division of germ cells, protein synthesis in the testis and induction of cell death in seminal vesicle. Gentamycin decreases sperm count, motility and viability. There were reports on inhibition of antioxidant enzymes, SOD

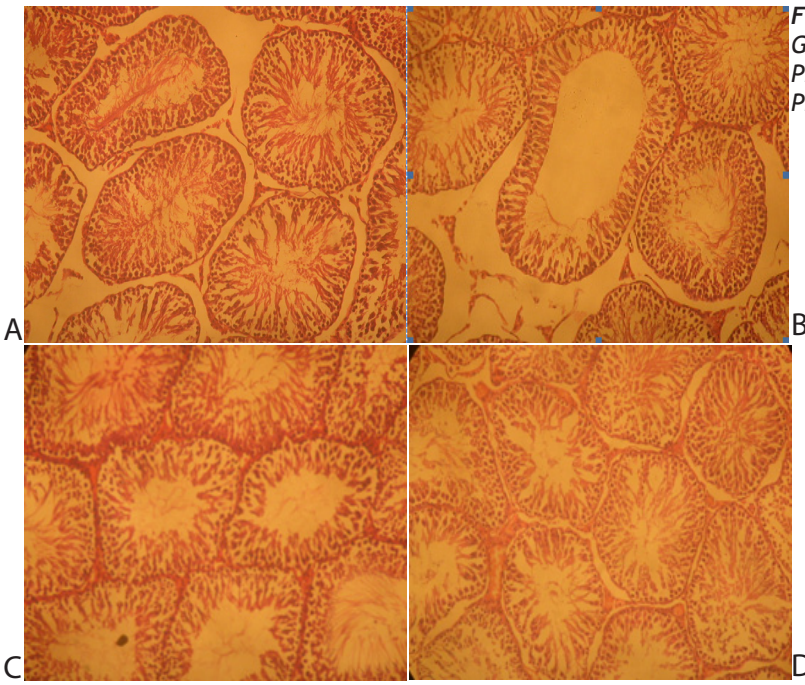

***Figure 7**. A: Sham control, B: After Gentamycin treatment-control, C: Protective effect of rutin 10mg/kg, D: Protective effect of naringin10mg/kg*

and catalase by the gentamycin treatment. Gentamycin was found to increase the tissue malanoldehyde levels. In general cellular damage in the semen is the result of an improper balance between ROS generation and scavenging activities. Excessive ROS production that exceeds critical levels can overwhelm all antioxidants defense strategies of spermatozoa and seminal plasma causing oxidative stress (9). Spermatozoa are highly susceptible to damage by excessive concentrations of ROS due to the high content of polyunsaturated fatty acids within their plasma membrane. The lipid peroxidation destroys the structure of lipid matrix in the membranes of spermatozoa, and it is associated with loss of motility and impairment of spermatogenesis. All the above reports clearly suggest the role of oxidative stress in gentamycin induced reproductive abnormalities (8).

As might be expected, inhibitors of oxidative stress provide significant beneficial effect on gentamycin induced testicular oxidative stress. Bioflavonoids, considered as classical example for antioxidants were already proved as effective agents in various models both in vitro and in vivo. Bioflavonoids, rutin and naringin are powerful radical scavengers, and their free radical scavenging ability may be due to their inhibitory activity on the enzyme xanthine oxidase (9). When oxygen is supplied during reperfusion, xanthine oxidase converts hypoxanthine to uric acid and superoxide radicals. Other flavonoids like Qurecetin and Silibin also inhibit xanthine oxidase activity, thereby resulting in decreased oxidative injury (22). Bioflavonoids were proved to inhibit degranulation of neutrophils and inhibition of arachidonic acid. Both Rutin and Naringin were proved to inhibit lipid peroxidation (11,13,23,24). Antioxidants protect DNA and other important molecules from oxidation and damage, and can improve sperm quality and consequently increase fertility rate in men. Several studies have reported that antioxidants and vitamin A, B, C, and E in diet can protect sperm DNA from free radicals and increase blood testes barrier stability (25,26).

In the present study, we have selected rutin and naringin and administered to the rats in two different doses, 5mg/kg and 10mg/kg. Biochemical parameters like MDA, SOD and catalase, sperm parameters like count, motility and viability were estimated after completion of 35 days treatment. In addition histopathology studies were also performed. Results indicate increase in MDA levels, reduction in antioxidant reserves SOD, Catalase in gentamycin treated control group animals when compared to the Sham control animals. Sperm count, motility and viability were significantly reduced in control group ani-

mals after gentamycin treatment (Table1). It indicates oxidative stress and its implications on testicular activity (Figure 1). Histopathological examinations were also significantly affected and degeneration of seminiferous tubules and necrosis was observed. The results are in accordance with the earlier reports on gentamycin (5). Vehicle treated animals (Group 3) shown similar results as group 2 (control). This shows that vehicle was not having any protective effect. Rutin and Naringin treated groups, 4 to 7 have shown dose dependent, significant decrease in MDA levels and increase in SOD, Catalase levels. Sperm count and percentages of sperm motility and viability were improved and become normal with higher doses of rutin and naringin. This might be due to acute antioxidant effect of bioflavonoids, rutin and naringin. Histopathological findings of the above groups also confirmed the same. There was improvement in testicular architecture in groups treated with rutin and naringin. Rutin had more antioxidant activity when compared to naringin, and was correlated to their structure. In fact rutin showed higher scavenger efficiency than naringin in DPPH, and TBA tests. This effect may be attributable to the catechol structure of ring B, the 2, 3 double bond in conjugation with a 4-oxo function, and the presence of both 7- and 5-hydroxyl groups (27).

To our knowledge no bioflavonoid was tested for its protective effect against gentamycin/antibiotic induced testicular oxidative stress. Bioflavonoids, rutin and naringin have elicited a significant and dose dependent protective role against gentamycin induced testicular oxidative stress. The antioxidant property may be attributed to their action on lipid peroxidation and attenuation of oxidative stress and increment in antioxidant reserves.

So, antioxidants rutin and naringin can be included in the prescription along with gentamycin, so that adverse effects of gentamycin on male reproductive system can be avoided.

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