Original Article

Ribavirin Exposure Induces Histopathological Changes in the Seminiferous Tubules of Testes in Albino Rats

Alvia Batool[1], Fozia Farzana[2]

ABSTRACT

Study objectives: The objectives of the study are to describe and compare histopathological changes in the seminiferous tubules of testes of rat, with different doses of Ribavirin at different time intervals.

Introduction: The chemical disturbances may affect a vast number of potential sites in male reproductive system as well as its complex hormonal regulation. Testicular toxicity may reduce the fertility of the male. The current study was conducted to evaluate the effects of Ribavirin on the histological structure of seminiferous tubules in the testes of albino rats.

Materials and Methods: Seventy two sexually mature adult male albino rats weighing 180-200gms were divided into four groups: A, B, C and D; each group having 18 rats. Ribavirin was administered intraperitoneally in different doses to these groups that were 20mg, 100mg and 200mg/kg body weight, while group A was control. Each group was further divided into three subgroups according to three time points which were selected for sacrifice that were 20th, 40th and 60th day from the last exposure to drug. Six randomly selected rats from each group were sacrificed on every sacrifice time.

Results and Conclusion: The seminiferous tubules with degenerative changes like appearance of vacuole and necrotic material were observed in comparison to control groups, on 20th day of sacrifice in all groups. In rats sacrificed on day 40th and 60th, the sign of recovery in the form of regeneration of seminiferous epithelium was observed that was more marked in low dose groups than high dose groups which showed late recovery. We conclude that ribavirin being used as antiviral drug induces reversible degenerative changes in the seminiferous tubules of testes of albino rats.

***Key words**: Testes, Rat, Cytotoxicity, Seminiferous germinal epithelium, Degeneration, Regeneration, Ribavirin.*

Alvia Batool, Assistant Professor in the Department of Anatomy, FMH College of Medicine & Dentistry, Lahore.

Fozia Farzana, Professor & Head of Anatomy Department in Postgraduate Medical Institute and Ameer-ud-Din Medical College, Lahore

INTRODUCTION

Ribavirin is a non-selective, antihepatitis, antiviral drug. [1] It was synthesized in 1970. The broad spectrum antiviral activity was reported in 1972. The aerosolic form was approved for the treatment of Respiratory Syncytial Virus in children. The ribavirin (orally) and Interferon alpha (injections) combination therapy was approved by United States regulatory Authorities in 1998 for the treatment of Hepatitis C infection. [2] Intravenous form reduces the mortality of Lassa and hemorrhagic fevers.[3,4]The chemical name of ribavirin is 1-beta-D-ribofurnosyl-1*H*-1, 2, 4-triazole-3-carboxamide. It is a purine (guanosine) nucleoside analog with modified base and d-ribose sugar, both are necessary for its antiviral activity. [5] Ribavirin has three metabolites Mono-, Di- and

Triphosphates that are effective against various RNA and DNA viruses. [6] Ribavirin-5' – triphosphate is the principal intracellular form.[7] Ribavirin exerts its cytotoxicity in the testes after intraperitoneal administration by getting absorbed from peritoneal cavity and reaching to the germ cells. It acts a germ cell mutagen in rats. It also inhibits the activity of IMPDH that possibly reduces the guanylate concentration resulting in decreased cell growth and may cause chromosomal damage.[8]

Weiss et al. reported that ribavirin administration in cats resulted in pathological changes including hepatocellular vacuolization and centrilobular necrosis.[9]

A study was conducted by Levine; he noticed that ribavirin not only reduced testes weight, sperm count, seminiferous tubular diameter and germinal epithelial thickening but increased the incidence of vacuolization and necrosis in mice.[10] D'Souza et al. while studying mechanism of ribavirin cytoxicity in testes of rats found dead cells and arrest of cell division in seminiferous tubules.[11] Ribavirin is reversibly cytotoxic to germ cells and decreases the production of sperms. Narayana et al. found a decrease in sperm count in a dose and time dependent pattern in the epididymis of rats receiving ribavirin.[8] He found it as mutagenic agent to germ cells in a transient fashion inducing anomalies of head and tail of sperms.[12] In humans, ribavirin was found reversibly genotoxic due to its toxic metabolites in patients of Crimean-Congao hemorrhagic fever treated with the therapeutic doses of the said drug.[13]

The present study is, therefore, designed to examine the effects of ribavirin on the histological architecture of seminiferous tubules of testes in rats as an experimental model with the hope that the results of this study may pave the way for reassurance of a patient using this drug about the reversibility of its gonadotoxic effects on his fertility. The usage of effective contraceptive measures during the treatment with the said drug must be advised.

MATERIALS AND METHODS

72 sexually mature adult male Wistar albino rats, weighing approximately 180 - 200gms were procured from the animal house of National Institute of Health (NIH), Islamabad. The animals were examined thoroughly for any pathology and weighed before commencement of experiment. The rats were housed at the animal house of Post Graduate Medical Institute, Lahore under optimum conditions of temperature 24±2˚C, humidity 50±10%, and in 12 hours light and 12 hours dark cycles. All animals were fed on normal rat chow and water ad libitum. After initial acclimatization of 5 days the rats were divided into four groups A, B, C and D, each group having 18 rats.[14] This division was done by using random number table. Ribavirin being used in this research is a product of Getz pharma company, Karachi, Pakistan. The dose of Ribavirin was in accordance with the protocol of Narayana, et al.[15] Procedure of dose calculation is given in Appendix I. Ribavirin was weighed on a scientific balance (Sartorius precision balance®, Germany) at PGMI, LHR. Ribavirin was dissolved in distilled water and was given at the dose levels of 20mg/kgb.w, 100mg/kgb.w and 200mg/kgb.w to the experimental animals of group B, group C and group D respectively. The drug was administered intraperitoneally using insulin-U-100 syringes, at 24 hrs interval for 5 consecutive days. Whereas control group A was given equal amounts of distilled water intraperitoneally at same time interval and for the same duration. At the 20th, 40th and 60th day, after the last exposure to the drug, six animals were randomly selected from each study group including control group and were sacrificed. Three subgroups of each study group, A, B, C and D were formed according to three sacrifice times, hence making 12 subgroups in total as shown in Table 1.

Table 1: EXPERIMENTAL CHART

Groups	*Subgroups*	*Dose of Ribavirin*	*Schedule of sacrifice days from the last dose*
Control A	A1 A2 A3	0.75ml distilled water	A1,20th day A2, 40th day A3, 60th day
Experimental B	B1 B2 B3	20mg/kg ribavirin dissolved in 0.75 ml distilled water.	B1, 20th day B2, 40th day B3, 60th day
Experimental C	C1 C2 C3	100mg/kg ribavirin dissolved in 0.75 ml distilled water.	C1, 20th day C2, 40th day C3, 60th day
Experimental D	D1 D2 D3	200mg/kg ribavirin dissolved in 0.75 ml distilled water.	D1, 20th day D2, 40th day D3, 60th day

Rats were weighed at the time of sacrifice and were anaesthetized. A vertical midline incision was given which was extended laterally to open the abdomen and thorax. The scrotum was cut longitudinally, epididymis was separated from testes surface and both testes were removed. Weight of each testis was recorded separately. Testes were fixed in Bouin's solution for 18hrs. The tissues were processed and embedded in liquid paraffin and blocks were prepared. Horizontal sections of 3-5 micrometer thickness were obtained by using a microtome. The slides were stained with Hematoxylin & Eosin, and studied under light microscope with X100 and X200 magnifications. Transversely cut seminiferous tubules were selected and changes in the cellular elements of seminiferous tubules including vacuolization, necrosis, cellular degeneration, cellular regeneration and sertoli cell changes were studied in comparison with controls and observations were recorded in tables 1, 2, 3.

STATISTICAL ANALYSIS

The data was entered and analyzed using SPSS 17.0 (Statistical Package for Social Sciences). The arithmetic mean of observations was calculated; standard deviation of mean values was calculated and the significance between two means was calculated by Pearsons chi square test for qualitative data at 5 % level of significance (taking p-value ≤ 0.05 as significant).

RESULTS AND OBSERVATIONS

On the 20th day from the last dose, seminiferous tubules of control group A1 were richly populated with all types of healthy looking germ cells (spermatogonia, spermatocytes, spermatids) and sertoli cells lying on a regular basement membrane surrounded by flattened nuclei of myoid cells. The lumen of the tubules can easily be was observed near the lumen. The lumen was devoid of sperms. These tubules also delineated and mature spermatozoa occupied most of it (Fig 1).

In experimental group B1, compared to control group A1 the majority of the tubules were shrunken in size and surrounded by wavy basement membrane with prominent flattened nuclei of myoid cells. Germinal epithelial cells showed degenerative changes as germ cells with pyknotic nuclei were observed, only healthy looking sertoli cells could be seen in the eosinophilic necrotic cellular debris filling the whole tubule. Many tubules contained few germ cells probably spermatogonia and large sized spermatocytes with pyknotic nuclei. An occasional multinucleated cell was observed near the lumen. The lumen was devoid of sperms. The tubules also showed necrotic cellular debris and vacuolization (Fig 1).

In C1 and D1 groups nearly all tubules were shrunken in size surrounded by wavy basement membrane with prominent flattened nuclei of myoid cells. The germinal epithelial cells showed degenerative changes with pyknotic nuclei, most of the cells degenerated, only sertoli cells with prominent nucleolus could be seen in the eosinophilic necrotic cellular debris filling the whole tubule. A small number of tubules were containing few spermatogonia, few large sized spermatocytes with pyknotic nuclei and an occasional multinucleated cell showed necrotic cellular debris and vacuolization in germinal epithelium (Fig 1).

On 40^{th} day from the last dose, seminiferous tubules of control group A2 were richly populated with all types of healthy looking germ cells and sertoli cells lying on a regular basement membrane. The lumen of the tubules can easily be delineated and mature spermatozoa occupied most of it (Fig 2). In experimental group B2 in comparison with control group A2 (Fig 2) Majority of the

seminiferous tubules grew in size and were repopulated with regenerated germ cell layers evidenced by mitotic figures and also having sertoli cells lying on a regular basement membrane. Some of the tubules were still shrunken in size surrounded by wavy basement membrane with prominent flattened nuclei of myoid cells. All types of germinal epithelial cells degenerated and disappeared, only sertoli cells could be seen in the eosinophilic necrotic cellular debris filling the whole tubule. Some of these tubules were containing one or two spermatogonia or spermatocytes with pyknotic nuclei could be observed near the lumen. The lumen was devoid of sperms. These tubules were showing cellular degeneration, necrotic cellular debris formation and vacuolization (Fig 2).

In C2 and D2 experimental groups as compared to A2 control group, majority of seminiferous tubules were showing necrotic changes, vacuolization and degenerative changes of germinal epithelium. Few large sized tubules in C2 group and very few in D2 group were showing regenerated germinal epithelium and sertoli cells with prominent nucleoli were resting on a regular basement membrane (Fig 2).

On 60th day from the last dose, seminiferous tubules of control group A3 were richly populated with all types of healthy looking germ cells and sertoli cells lying on a regular basement membrane. The lumen of the tubules can easily be delineated and mature spermatozoa occupied most of it (Fig 3). B3 study group showed that all seminiferous tubules had gained large size and were richly populated with all types of regenerated germ cells and sertoli cells were resting on a regular basement membrane in comparison to A3. These tubules were also showing mitotic figures. That means this group was showing full recovery and regeneration of germ cells inside the tubules (Fig 3). In medium and high dose groups C3 and D3 respectively, few tubules were large sized richly populated with all types of germ cells and sertoli cells lying on a regular basement membrane, showing regeneration of germinal epithelium evidenced by the presence of mitotic figures. Majority of the tubules among regenerated tubules were small sized, distorted and wavy in outline showing vacuolization, necrosis and degenerative changes of germ cells. Their lumina were devoid of sperms (Fig 3). A comparison between experimental and control groups at various time points was done by applying Chi square test for necrosis, vacuolization, degeneration and regeneration inside the seminiferous tubules. It was found to be highly significant (p-value <0.001) in all study groups (Table 1, 2, 3). Sloughing (Separation of germinal epithelium from basement membrane) was not observed with any dose level, at any sacrifice time. So Chi square test was invalid for it.

Table No 1: Comparison of A1, B1, C1 and D1 groups for the presence of histopathological changes in the seminiferous tubules on 20th day from the last dose

Variables	A1	B1	C1	D1	df	p-value
	n(%)	n(%)	n(%)	n(%)		
Sloughing	00(0.0)	00(0.0)	00(0.0)	00(0.0)	-	-
Vacuolization	00(0.0)	6(100)	6(100)	6(100)	3	0.000*
Necrosis	00(0.0)	6(100)	6(100)	6(100)	3	0.000*
Degeneration	00(0.0)	6(100)	6(100)	6(100)	3	0.000*
Regeneration	00(0.0)	00(0.0)	00(0.0)	00(0.0)	-	-

*- p-value < 0.05 statistically significant
**- p-value < 0.005 highly significant

Table No 2: Comparison of A2, B2, C2 and D2 groups for the presence of histopathological changes in the seminiferous tubules on 40th day from the last dose

Variables	A2	B2	C2	D2	df	p-value
	n(%)	n(%)	n(%)	n(%)		
Sloughing	00(0.0)	00(0.0)	00(0.0)	00(0.0)	-	-
Vacuolization	00(0.0)	6(100)	6(100)	6(100)	3	0.000**
Necrosis	00(0.0)	6(100)	6(100)	6(100)	3	0.000**
Degeneration	00(0.0)	6(100)	6(100)	6(100)	3	0.000**
Regeneration	00(0.0)	6(100)	6(100)	6(100)	3	0.000**

*- p-value < 0.05 statistically significant
**- p-value < 0.005 highly significant

Table No 3: Comparison of A3, B3, C3 and D3 groups for the presence of histopathological changes in the seminiferous tubules on 60th day from the last dose

Variables	A3	B3	C3	D3	df	p-value
	n(%)	n(%)	n(%)	n(%)		
Sloughing	00(0.0)	00(0.0)	00(0.0)	00(0.0)	-	-
Vacuolization	00(0.0)	00(0.0)	6(100)	6(100)	3	0.000**
Necrosis	00(0.0)	00(0.0)	6(100)	6(100)	3	0.000**
Degeneration	00(0.0)	00(0.0)	6(100)	6(100)	3	0.001**
Regeneration	00(0.0)	6(100)	6(100)	6(100)	3	0.000**

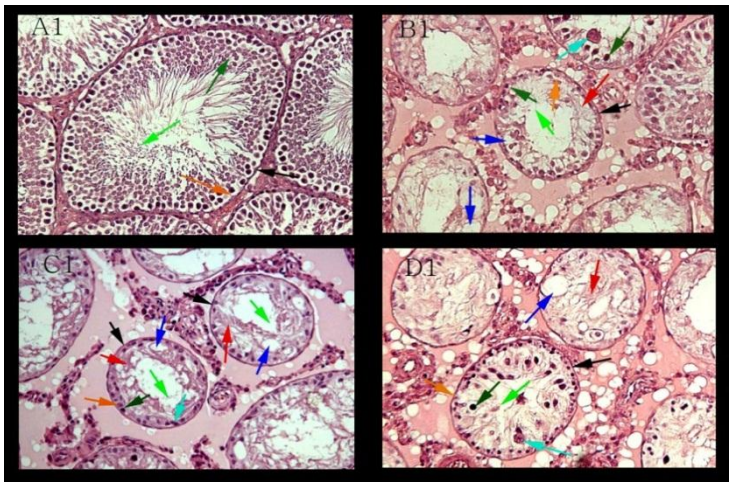

Figure 1: A Photomicrograph of testis of rat from control group A1, showing seminiferous tubule with regular basement membrane surrounded by flattened nuclei of myoid cells (Black arrow), germinal epithelium (Dark green arrow), Sertoli cells (Orange arrow) and lumen filled with sperms (light green arrow). H&E. X100.

Photomicrographs of testes of rats from B1, C1 and D1 experimental groups, showing shrunken seminiferous tubules having wavy outline with prominent nuclei of myoid cells (Black arrow), few germ cells with pyknotic nuclei (Dark green arrow), empty lumen (Light green arrow), Sertoli cells (Orange arrow), pink necrotic material (Red arrow), Vacuolization (Royal blue arrow) and a multinucleated cell (Cyan blue arrow). H&E. X100.

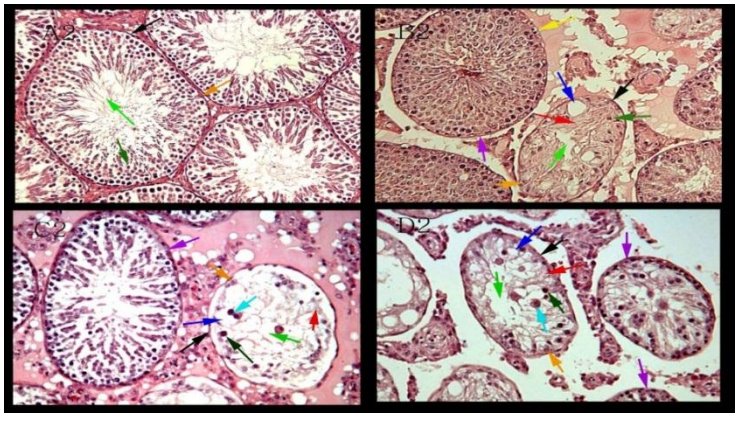

Figure 2: A Photomicrograph of testis of rat from control group A2, showing seminiferous tubule with regular basement membrane surrounded by flattened nuclei of myoid cells (Black arrow), germinal epithelium (Dark green arrow), Sertoli cells (Orange arrow) and lumen filled with sperms (light green arrow). H&E. X100.
Photomicrograph of testis of rat from experimental group B2, showing a shrunken seminiferous tubule having wavy outline with prominent nuclei of myoid cells (Black arrow), few germ cells with pyknotic nuclei (Dark green arrow), empty lumen (Light green arrow), Sertoli cells (Orange arrow), pink necrotic material (Red arrow), Vacuolization (Royal blue arrow). Many seminiferous tubules filled with regenerated epithelium (Purple arrow) and mitotic figures(Yellow arrow) seen. H&E. X100.
Photomicrographs of testes of rats from experimental groups C2 and D2, showing same picture of necrosis & degeneration in seminiferous tubules as B2 study group. In addition, Multinucleated cells seen (Cyan blue arrow). One Seminiferous tubule in each group with regenerating epithelium seen (Purple arrow). H&E. X100.

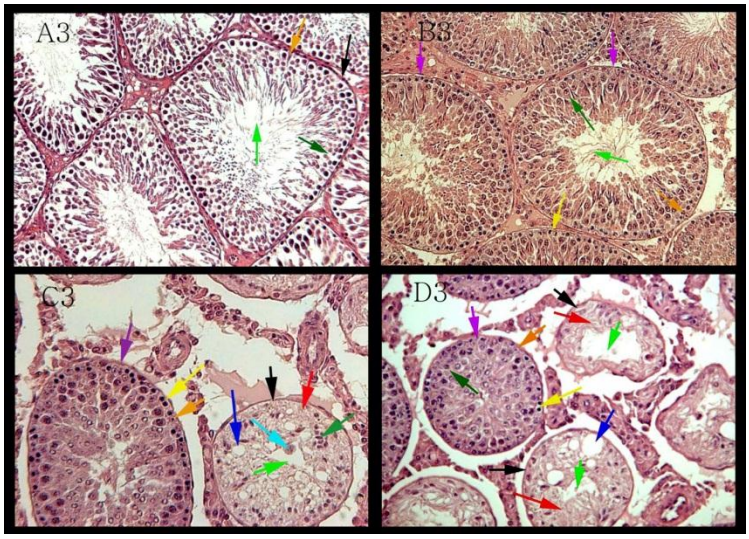

Figure 3: A Photomicrograph of testis of rat from control group A3, showing seminiferous tubule with regular basement membrane surrounded by flattened nuclei of myoid cells (Black arrow), germinal epithelium (Dark green arrow), Sertoli cells (Orange arrow) and lumen filled with sperms (light green arrow). H&E. X100.
Photomicrographs of testis of rat from experimental group B3, showing all seminiferous tubules with regenerated germ cells (Dark green arrow), lumen filled with sperms (Light green arrow), sertoli cells (Orange arrow) and mitotic figures (Yellow arrow). H&E. X100.
Photomicrographs of testes of rats from experimental groups C3 and D3, showing many shrunken seminiferous tubules having wavy outline with prominent nuclei of myoid cells (Black arrow), few germ cells with pyknotic nuclei (Dark green arrow), empty lumen (Light green arrow), Sertoli cells (Orange arrow), pink necrotic material (Red arrow), Vacuolization (Royal blue arrow), Multinucleated cells (Cyan blue arrow). One Seminiferous tubule in each group filled with regenerated epithelium (Purple arrow) and mitotic figures (Yellow arrow) seen. H&E. X100.

DISCUSSION:

Regarding the histology of seminiferous tubules, on the day 20th from the last dose of ribavirin seminiferous tubules were noted with wavy outlines surrounded by prominent nuclei of myoid cells all around and depletion of spermatogonia and spermatocytes with empty lumina of tubules devoid of spermatids and

sperms. Sertoli cells seemed to be unaffected. In addition there was cellular debris (necrosis) inside the tubules with occasional multinucleated cells specifically seen on 20th and 40thdays from the last dose of drug and the germinal epithelium was showing degeneration and vacuolization. The inflammatory cells were not seen. These changes were statistically significant (p-value< 0.001) (Tables 1, 2, 3) at all time points. A gradual reduction in the qualitative changes of seminiferous tubules and recovery were observed as time passed, more marked in low dose group. After that on the day 40th, in addition to the presence of cellular debris, degenerative changes, vacuolization in the germinal epithelium with the presence of occasional multinucleated cells there was presence of regenerated tubules with marked regeneration evidenced by mitotic figures. Regeneration was more marked in low dose group. All changes in germinal epithelium were statistically significant in all experimental groups (p-value<0.001) (Tables 1, 2, 3). On 60th day from the last dose of ribavirin germ cell population increased and filled the tubules completely in group B3 low dose group. The higher dose groups were having tubules with necrosis, degeneration and vacuolization along with few tubules showing regeneration of germinal epithelium. All these changes were statistically significant (p-value<0.001) (Tables 8, 9, 10).

These findings are comparable to the study outcomes of (d) Narayana, etal. Who confirmed that ribavirin induced formation of vacuoles and gaps in the seminiferous epithelium in all dose groups at all sacrifice times, although the reversibility was observed except two higher dose groups. The vacuoles are due to non specific injury to germ cells.[15] Necrosis by ribavirin could be in the form of pinkish cellular debris without any inflammatory cells, it was suggested by Ning, et al. that ribavirin is a very potent inhibitor of virus induced proinflammatory mediators.[16]Meier, et al. noticed that ribavirin administered to HCV patients resulted in a decrease in the synthesis of proinflammatory cytokines by an inhibition of total DNA-, RNA- and protein-synthesis, and also caused induction of apoptosis in the cells of inflammatory infilterate.[17] Levine, etal. observed similar transient findings of vacuolization, necrosis along with decrease in seminiferous tubular diameter and germinal epithelial height in the testes of mice after ribavirin administration.[10] Ribavirin deplete the sperms, this finding is in accordance to D'Souza, et al. who observed that cytotoxicity of ribavirin was imparted on rapidly dividing target tissue where cells were removed by cell death somewhat similar to apoptosis and it was responsible for decrease in erythrocytes and sperm counts in rats.[11] Testicular histopathology is considered the most sensitive parameter for the detection of testicular toxicity, which should be assessed for the presence of cellular necrosis, giant cell formation and other degenerative changes.[18] Mulinucleated giant cells are composed of degenerated spermatocytes or spermatids. Giant cell formation is the result of failure of intercellular bridges formed during germ cell division to constrict or they open up due to an insult to testicular tissue. As a consequence of this type of insult multinucleated syncytia of spermatocytes and spermatids are formed.[19] Mutinucleate giant cells are commonly seen in degenerated seminiferous tubules of many species.[20]In this study no apparent changes in Sertoli cells were observed indicating that the tubular degeneration was not due to these cells. As Proliferating cells are particularly sensitive to mutagenic agents. Non dividing Sertoli cells, Leydig cells and reserve stem cells which demonstrate low mitotic activity are much less vulnerable than actively dividing and differentiating spermatogenic cells.[21] Cytotoxic agents cause gonadal dysfunction. The process of spermatogenesis is much more affected than is the testosterone production. The underlying reason is, the germinal epithelium of testis is more sensitive to the damage caused by these agents than the Leydig cells.[22] Due to shrinkage of seminiferous tubules owing to the germ cell loss, myoid cell nuclei became more prominent.

CONCLUSION:

This study showed that ribavirin given to albino rats exerted toxic and degenerative effects on the histological architecture of testes with dose related and time related recovery. Only the low dose group showed recovery to greater extent, although high dose treated groups showed little

recovery by the end of this study. The higher dose treated groups must be studied for longer time period till the testicular tissue show full recovery. The physicians while prescribing this drug to the patients must consider its possible gonadotoxic effects on his fertility. Reassurance of a patient is necessary using this drug about the reversibility of its effects. The usage of effective contraceptive measures during the treatment should be advised. The pathologists must consider the history of ribavirin intake while diagnosing a testicular pathology if they observe the same histopathological picture in the biopsy specimen of the testes of a patient.

REFERENCES:

1. Trevors JA, Katzung BG, Masters SB. Antiviarl chemotherapy and prophylaxis. In: Katzung & Trevor's pharmacology. 9th ed. United States: McGraw Hill companies, 2010. p. 434-438.
2. Lau JYN, Tam RC, Liang TJ, Hong Z. Mechanism of action of ribavirin in the combination treatment of chronic HCV infection. Hepatology. 2002; 35 (5): 1002-1009.
3. Ali KN, Anushree J, Reenu C, Mohammad A, Kamal K. Crimean Congo hemorrhagic fever- An article review. International research journal of pharmacy. 2011; 2 (5): 33-40.
4. Acosta EP, Flexner C. Antiviral agents (Non retroviral). In: Brunton LL, Chabner BA, Knollmann BC, editors. Goodman and Gilman's. The pharmacological basis of Therapeutics. 12thed. New York: Mcgraw Hill Companies; 2011. p. 1613-1615.
5. El-Brashy AM, Sheribah ZA, Sharaf El-Din MK, El-Gamal RM. Kinetic determination of ribavirin in drug formulations. International Journal of biomedical sciences. 2007; 3 (1): 65-71.
6. Preston SL, Drusano GL, Glue P, Nash J, Gupta SK, Mcnamara P. Pharmocokinetics and absolute bioavailability of ribavirin in healthy volunteers as determined by stable-isotope methodology. AntiMicrob Agents and Chemother. 1999; 43 (10): 2451-2456.
7. SIS: Chem ID plus record for Ribavirin (36791-04-5) [Internet]. National library of Medicine; 2006 (Cited 2011 December 10) Available from: http://chem.sis.nlm.nih.gov/chemidplus/chemidlite.jsp National Toxicology program-CAS Registry number: 36791-04-5. Toxicity effects.
8. b)Narayana K, D'Souza UJA, Rao KP. Effect of ribavirin on epididymal sperm count in rat. Indian J physiol Pharmacol. 2002; 46 (1): 97-101.
9. Weiss RC, Cox NR, Boudreaux MK. Toxicologic effects of ribavirin in cats. Journal of veterinary pharmacology and Therapeutics. 1993; 16 (3): 301-316.
10. Levine BS, Tomlinson MJ, Parker RM. Onset and reversibility of ribavirin-induced testicular toxicity in mice. Toxic subst j. 1994; 13 (3); 171-186.
11. D'Souza UJ, Narayana K. Mechanism of cytotoxicity of ribavirin in the rat bone marrow and testis. Indian. J. physiol. Pharmacol., 2002; 46 (4): 468-474.
12. c)Narayana K, D'Souza UJ, Rao KPS. Ribavirin-induced sperm shape abnormalities in Wistar rat. Mutat Res. 2002; 513 (1-2): 193-196.
13. Tatar A, Ozkurt Z, Hacimuftuoglu A, Yesilyurt A, Vancelik S. Evaluation of Ribavirin genotoxicity with sister chromatid exchange and micronuclei assays in humans. Turk J med Sci. 2009; 39 (2): 241-246.
14. Conour LA, Murray KA, Brown MJ. Preparation of animals for research-issues to consider for rodents and rabbits. ILAR Journal. 2006; 47 (4): 283-293.
15. d)Narayana K, D'Souza UJA, Narayan P. The antiviral drug ribavirin reversibly affects the reproductive parameters in male wistar rat. Folia.Morphol. 2005; 64 (2): 65-71.
16. Ning Q, Brown D, Parodo J, Cattral M, Gorczynski R, Cole E, Fung L, et al. Ribavirin inhibits viral-induced macrophage production of TNF, IL-1,

the procoagulant fgl2 prothrombinase and preserves Th1 cytokine production but inhibits Th2 cytokine response. The Journal of Immunology. 1998; 160: 3487-3493.

17. Meier V, Bürger E, Mihm S, Saile B, Ramadori G. Ribavirin inhibits DNA, RNA, and protein synthesis in PHA-stimulated human peripheral blood mononuclear cells: possible explanation for therapeutic efficacy in patients with chronic HCV infection. J.Med. Virol., 2003; 69 (1): 50-58.
18. Foley GL. Overview of male reproductive pathology. Toxicol.Pathol., 2001; 29 (1): 49-63.
19. Corrier DE, Mollenhauer HH. Clark DE, Hare MF, Elissalde MH. Testicular Degeneration and Necrosis Induced by Dietary Cobalt. Vet. Pathol., 1985; 22: 610-616.
20. Ackermann MR,etal. Light microscopic and ultrastructural pathology of seminiferous tubules of rats given multiple doses of pasteurella multocida Group D protein toxin. Toxicologic pathology, 1992; 20 (1): 103-111.
21. Ross, M.H. and Pawlina, W. Male reproductive system. In: Histology: A text and Atlas with correlated cell and molecular biology. 5th ed. Philadelphia.USA: Lippincott Williams and Wilkins, 2006; p. 742.
22. Hayes FJ, Bubley JG. Effect of cytotoxic agents on gonadal function in adult men. [Internet] Walter and Kluwer Health; 2012 (Cited 2012 August 30) Available from: http://www.update.com/cntents/effects-of-cytotoxic-agents-on-gonadal-function-in-adult-men.