RIBAVIRIN EXPOSURE INDUCES MORPHOMETRIC CHANGES IN THE SEMINIFEROUS TUBULES OF TESTES IN ALBINO RATS

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ABSTRACT

Study Objectives: The objectives of the study were to describe and compare, seminiferous tubular diameter (STD) and seminiferous epithelial height (SEH) 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 effect of Ribavirin on the tubular diameter and epithelial height of seminiferous tubules in the testes of albino rats.

Materials and Methods: Seventy two sexually mature adult male albino rats weighing 180 – 200 gms 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 20 mg, 100 mg and 200 mg/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 every time.

Results and Conclusion: A decrease in the values of seminiferous tubular diameter and epithelial height of seminiferous tubules was noticed in comparison to control groups, on 20th day of sacrifice in all experimental groups. In rats sacrificed on day 40th and 60th, the signs of recovery were observed that were 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, Gonadotoxicity, Seminiferous tubular diameter, Seminiferous epithelial height, Ribavirin.

INTRODUCTION

Ribavirin is a non selective, anti-hepatitis, 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 haemorrhagic 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 intra-peritoneal administration by getting absorbed from peritoneal cavity and reaching the germ cells. It acts as 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]

A study was conducted by Levine; he noticed that Ribavirin reduces testes weight, sperm count, seminiferous tubular diameter and germinal epithelial thickness in mice.[9] Ribavirin is reversibly cytotoxic to germ cells and decreases the production of sperms. Narayana 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.[10] 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.[11]

The present study is, therefore, designed to examine the effects of ribavirin on the morphometry

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.

Table 1: *Experimental Chart.*

Groups	*Sub-groups*	*Dose of Ribavirin*	*Schedule of Sacrifice days from the Last Dose*
Control A	A_1 A_2 A_3	0.75 ml distilled water	A1,20th day A2, 40th day A3, 60th day
Experimental B	B_1 B_2 B_3	20 mg/kg ribavirin dissolved in 0.75 ml distilled water.	B1, 20th day B2, 40th day B3, 60th day
Experimental C	C_1 C_2 C_3	100 mg/kg ribavirin dissolved in 0.75 ml distilled water.	C1, 20th day C2, 40th day C3, 60th day
Experimental D	D_1 D_2 D_3	200 mg/kg ribavirin dissolved in 0.75 ml distilled water.	D1, 20th day D2, 40th day D3, 60th day

MATERIALS AND METHODS

Seventy two sexually mature adult male Wistar albino rats, weighing approximately 180 – 200 gms 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.[12] 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.[13] 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 20 mg/kgb.w, 100 mg/kgb.w and 200 mg/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.

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 and Eosin, and studied under light microscope. STD and SEH were measured using ocular micrometer under X100 magnification. Transversely cut seminiferous tubules were selected, in each tubule both maximum and minimum diameters were measured and their average was taken. The same tubules were considered to measure SEH from the basement membrane to surface of the epithelium at two locations and mean was taken.

Statistical Analysis

The data was entered and analyzed using SPSS 17.0. The arithmetic mean of observations was calculated; standard deviation of mean values was calculated and the significance between two means was calculated by Analysis of variance (ANOVA) and Post Hoc (Tukey) test for quantitative differences between mean groups at 5% level of significance (taking p-value ≤ 0.05 as significant).

RESULTS AND OBSERVATIONS

Seminiferous tubular diameter (STD)

On 20th day from the last treatment, the mean STD of testes of control group A_1 was 357 ± 34.44 that reduced to 222 ± 7.52 in low dose group B1 and the values of STD further reduced to 190 ± 1.26 and 180 ± 10.95, in medium dose group C_1 and high dose group D_1, respectively. Overall reduction in the values was compared by applying ANOVA, that was significant with p-value < 0.001. Post Hoc test showed statistically significant difference between all study groups. The test revealed no significant difference

between C_1 and D_1 groups (Table 2).

The mean values of Seminiferous tubular diameter (STD) were found reduced in all experimental groups, on the 40th day from the last dose. But this reduction was not as marked as observed on 20th day from the last treatment sacrifice. The values of STD in control group A2 was 345 ± 12.24, in low dose group B_2 it was 257 ± 5.23, in medium dose group C2 and high dose group D_2, these were 239 ± 5.54 and 234 ± 3.76, respectively. Overall reduction in the values was compared by applying ANOVA, that was significant with p-value < 0.001. Post-Hoc test showed statistically significant difference between all study groups. The test did not reveal any significant difference between C_2 and D_2 groups (Table 2).

The mean values of seminiferous tubular diameter (STD) showed reduction in all experimental groups, on the 60th day from the last dose. But this reduction was not as marked as observed on the 20th day and 40th day from the last treatment. The values of STD in control group A3 was 320 ± 14.14, in low dose group B3 it was 311 ± 16.02 that seemed to be very close to the value of control group. The mean values of STD in medium dose group C_3 and high dose group D3 were 277 ± 1.22 and 258 ± 2.73, respectively. This reduction in the values was compared by applying ANOVA, that was significant with p-value < 0.001. Therefore

Table 2: *Comparison of mean values of seminiferous tubular diameter (STD) in micrometer in study groups at different sacrifice times.*

Times	*Groups*	*STD µm*	*Anova*	*Post Hoc Test*	
		Mean ± SD	*p-value*	*Comparison Groups*	*STD p-value*
20th day	A_1	357 ± 34.44	<0.001**	$A_1 – B_1$	<0.001**
	B_1	222 ±7.52		$A_1 – C_1$	<0.001**
	C_1	190 ± 1.26		$A_1 – D_1$	<0.001**
	D_1	180 ± 10.95		$B_1 – C_1$	0.035**
				$B_1 – D_1$	0.004**
				$C_1 – D_1$	0.785
40th day	A_2	345 ± 12.24	<0.001**	$A_2 – B_2$	<0.001**
	B_2	257 ± 5.23		$A_2 – C_2$	<0.001**
	C_2	239 ± 5.54		$A_2 – D_2$	<0.001**
	D_2	234 ± 3.76		$B_2 – C_2$	0.002**
				$B_2 – D_2$	<0.001**
				C_2- D_2	0.74
60th day	A_3	320 ± 14.14	<0.001**	$A_3 – B_3$	0.55
	B_3	311 ± 16.02		$A_3 – C_3$	<0.001**
	C_3	277 ± 1.22		$A_3 – D_3$	<0.001**
	D_3	258 ± 2.73		$B_3 – C_3$	<0.001**
				$B_3 – D_3$	<0.001**
				$C_3 – D_3$	0.03*

*-p-value≤0.05 statistically significant change
**-p-value<0.005 highly significant change
STD: Seminiferous tubular diameter SD: Standard deviation

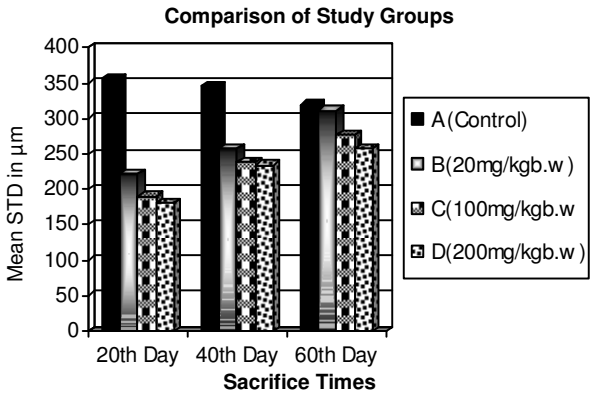


Fig. 1: *Bar Graph showing a comparison of Mean seminiferous tubular diameter (STD) between study groups at different times of sacrifice.*

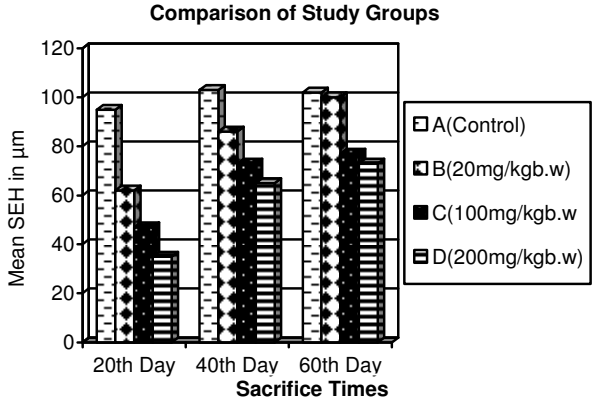


Fig. 2: *Bar Graph showing a comparison of Mean seminiferous epithelial height (SEH) between study groups at different times of sacrifice.*

Post-Hoc test was applied, that showed statistically significant difference between all study groups. The test revealed no significant difference between A_3 and B_3 groups due to recovery (Table 2). The seminiferous tubules of all study groups with changes in diameters at various sacrifice times are shown in figures 3, 4 and 5.

Table 3: *Comparison of mean values of seminiferous epithelial height (SEH) in micrometer in study groups at different sacrifice time.*

Times	*Groups*	*SEH µm*	*Anova*	*Post Hoc Test*	
		Mean ±SD	*p-value*	*Comparison Groups*	*SEH p-value*
20th day	A_1	95 ± 5.47	<0.001**	$A_1 - B_1$	<0.001**
	B_1	62 ±7.52		$A_1 - C_1$	<0.001**
	C_1	47 ± 5.16		$A_1 - D_1$	<0.001**
	D_1	35 ± 5.47		$B_1 - C_1$	0.002**
				$B_1 - D_1$	<0.001*
				$C_1 - D_1$	0.015**
40th day	A_2	103 ± 8.16	<0.001**	$A_2 - B_2$	<0.001**
	B_2	86 ± 3.76		$A_2 - C_2$	<0.001**
	C_2	73 ± 2.73		$A_2 - D_2$	<0.001**
	D_2	65 ± 5.47		$B_2 - C_2$	0.002**
				$B_2 - D_2$	<0.001**
				$C_2 - D_2$	0.11
60th day	A_3	102 ± 7.52	<0.001**	$A_3 - B_3$	0.98
	B_3	100 ± 12.64		$A_3 - C_3$	<0.001**
	C_3	77 ± 5.16		$A_3 - D_3$	<0.001**
	D_3	73 ± 5.16		$B_3 - C_3$	<0.001**
				$B_3 - D_3$	<0.001**
				$C_3 - D_3$	0.89

*-p-value≤0.05 statistically significant change
**-p-value≤0.005 highly significant change
SEH: Seminiferous epithelial height SD: Standard deviation

Seminiferous epithelial height (SEH)

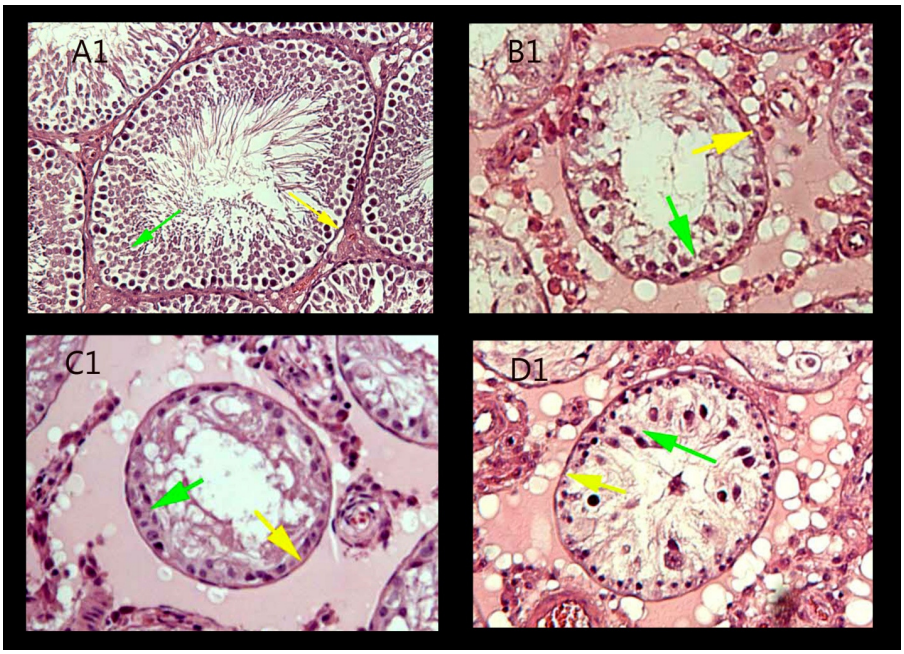
The mean SEH of testes of control group A_1 on 20th day from the last dose was 95 ± 5.47, that reduced to 62 ± 7.52 in low dose group B_1 and the values of SEH further reduced to 47 ± 5.16 and 35 ± 5.47, in medium dose group C_1 and high dose group D_1, respectively. Overall reduction in the values was compared by applying ANOVA, that was significant with p-value<0.001. Therefore Post-Hoc test was applied, that showed statistically significant difference between all study groups in all possible combinations (Table 2).

The mean values of Seminiferous epithelial height (SEH) were found reduced in all experimental groups, on the 40th day from the last treatment. But this reduction was not as marked as observed on 20th day from the last dose. The values of SEH in control group A_2 was 103 ± 8.16, in low dose group B_2 it was 86 ± 3.76, in medium dose group C_2 and high dose group D_2, these were 73 ± 2.73 and 65 ± 5.47, respectively. Overall reduction in the values was compared by applying ANOVA, that was significant with p-value < 0.001. Therefore Post-Hoc test was applied, that showed statistically significant difference between all study groups in all possible combinations. The test did not reveal any significant difference between C_2 and D_2 groups (Table 3).

The mean values of seminiferous epithelial height (SEH) showed reduction in all experimental groups, on the 60th day from the last treatment. But this reduction was not as marked as observed on the 20th day and 40th day from the last dose. The values of SEH in control group A_3 was 102 ± 7.52, in low dose group B_3 it was 100 ± 12.64 that seemed to be very close to the value of SEH in control group. In medium dose group C_3 and high dose group D_3, the values of SEH were 77 ± 5.16 and 73 ± 5.16, respectively. This reduction in the values was compared by applying ANOVA, that was significant with p – value < 0.001. Therefore Post-Hoc test was applied, that showed statistically significant difference between all study groups. The test revealed no significant difference between A_3 and B_3, C_3 and D_3 groups (Table 3). The seminiferous tubules of all study groups with changes in epithelial height at various sacrifice times are shown in figures 3, 4 and 5.

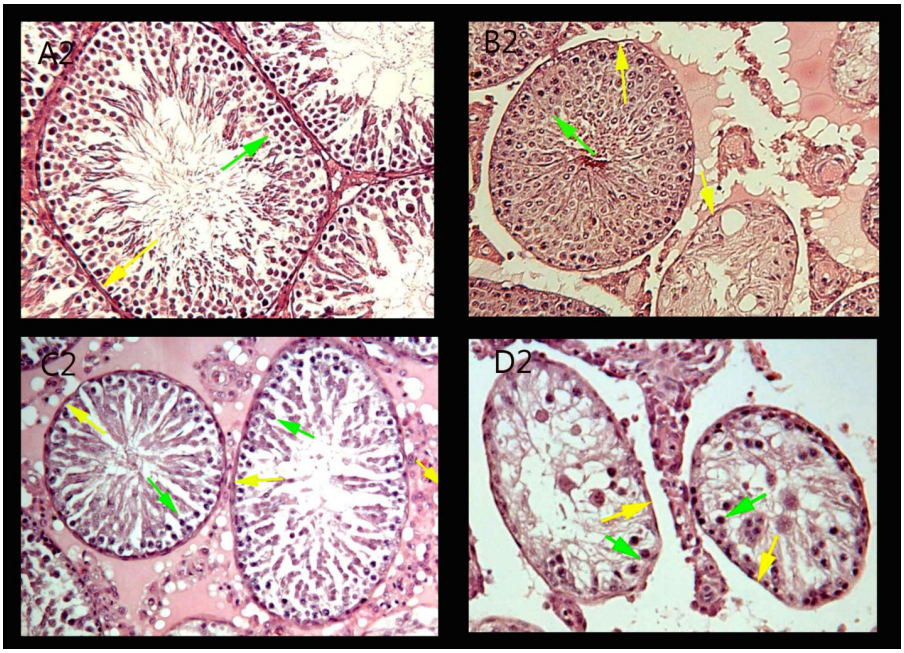
DISCUSSION

Regarding seminiferous tubular diameter STD, Ribavirin caused decrease in STD at all sacrifice times. When results were compared with control groups these were statistically significant (p-value <0.05) (Table 2) (Fig. 1) at all time points. However, no significant difference was found between C_1 and D_1 groups on 20th day (p-value > 0.05), C_2 and D_2 groups



Photomicrographs of testis of rat from B_1, C_1 and D_1 experimental groups, showing shrunken seminiferous tubules with wavy basement membrane (Yellow arrow) and few degenerating seminiferous epithelial cells (Green arrow). H&E. X100.

Fig. 3: *A Photomicrograph of testis of rat from A1 (Control) group, showing seminiferous tubule with normal basement membrane (Yellow arrow) and normal epithelium (Green arrow). H&E. X100.*



A Photomicrograph of testis of rat from B_2 experimental group, showing seminiferous tubule with recovery in size having regular basement membrane (Yellow arrow) and regenerated epithelial cells (Green arrow). H&E. X100.
A photomicrograph of testis of rat from C_2 experimental group, showing seminiferous tubules with little recovery in size having regular basement membrane (Yellow arrow) and regenerating epithelial cells (Green arrow). H&E. X100.
A photomicrograph of testis of rat from D_2 experimental group, showing shrunken seminiferous tubules with wavy basement membrane (Yellow arrow) and few degenerating Seminiferous epithelial cells (Green arrow). H&E. X100.

Fig. 4: *A Photomicrograph of testis of rat from A2 (Control) group, showing seminiferous tubule with normal basement membrane (Yellow arrow) and normal epithelium (Green arrow). H&E. X100.*

on 40th day (p-value > 0.05). Insignificant results were also noted between A_3 and B_3 groups (p-value > 0.05) on the 60th day from the last dose of the drug. Group C and D both were high doses, although there was some difference between their STD but it was not statistically significant. The only group that recovered completely was B_3 after lot of degenerative changes in the seminiferous tubules caused by ribavirin as indicated by (d) Narayana, et al.[11]

Seminiferous epithelial height SEH also showed reduction in all experimental groups after treatment with Ribavirin. The values were statistically signifi-

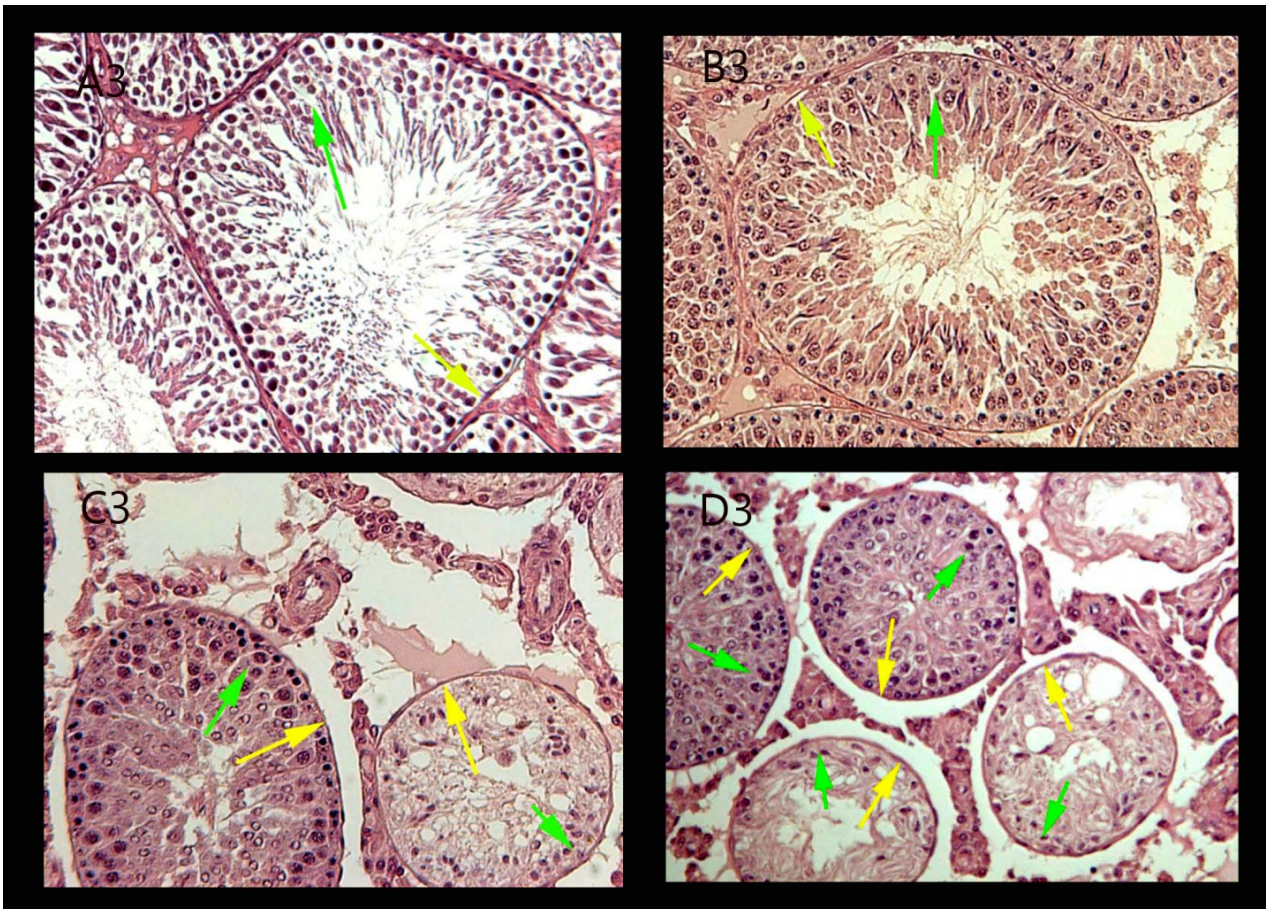


Fig. 5: *A Photomicrograph of testis of rat from A_3 (Control) group, showing seminiferous tubule with normal basement membrane (Yellow arrow) and normal epithelium (Green arrow). H&E. X100.*
A Photomicrograph of testis of rat from B_3 experimental group, showing seminiferous tubule with almost complete recovery in size having regular basement membrane (Yellow arrow) and regenerated epithelial cells (Green arrow). H&E. X100.
A photomicrograph of testis of rat from C_3 experimental group, showing seminiferous tubules with little recovery in size having regular basement membrane (Yellow arrow) and regenerating epithelial cells (Green arrow). A tubule with degenerated epithelium can be seen. H&E. X100.
A photomicrograph of testis of rat from D_3 experimental group, showing more shrunken seminiferous tubules with wavy basement membrane (Yellow arrow) and few Seminiferous epithelial cells (Green arrow). Seminiferous tubules with regenerated epithelium can be seen. H&E. X100.

cant (p-value < 0.05) (Table3) (Fig. 2) when compared with control group at all sacrifice times. The difference was insignificant between C_2 and D_2 groups (p-value > 0.05) on 40th day and between C_3 and D_3 groups (p-value > 0.05) on 60th day from the last dose of ribavirin. Insignificant difference was also observed between A_3 and B_3 groups (p-value > 0.05). Group C and D both were high doses, so they showed nearly similar changes. The only group that recovered completely was B_3 after lot of degenerative changes caused by ribavirin. These findings were confirmed by (d) Narayana, et al. who noticed that STD reduced on day 14 and 35 with low dose and two higher doses reduced the STD and SEH on day 35 and further on day 70th. All these changes were transitory. This decrease in STD and SEH might be due to spermatogenic arrest and germ cell depletion inside the seminiferous tubules. The decrease in STD and SEH might have occurred in positive correlation with the degenerative and necrotic changes in the germinal epithelial cells, indicating the shrinkage of seminiferous tubules as a result of cell loss.[13]

The results of this study are consistent with the findings of Levine et al, who evaluated reversible spermatotoxicity of ribavirin in testes of mice. He noticed that this particular drug caused reduction in seminiferous tubular diameter and germinal epithelial thickening of seminiferous tubules in the testes of mice.[9]

It is *concluded* that ribavirin given to albino rats

exerted toxic effects and degenerative effects on the STD and SEH 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 tumor if they observe the same histopathological picture in the biopsy specimen of the testes of a patient.

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