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Toxic Effects of Ribavirin on the Testicular Interstitium in Albino Rats

[1]Alvia Batool, [2]Maryam Fatima, [3]Fozia Farzana
[1]Department of Anatomy, FMH College of Medicine & Dentistry, Lahore
[2]Department of Neurology, FMH College of Medicine & Dentistry, Lahore
[3]Department of Anatomy, Continental Medical College, Lahore

ABSTRACT

Introduction: Ribavirin due to its mutagenic property exerts cytotoxic effects on gonads in a transient fashion that have been studied on the testicular tissues of various experimental animals. Ample data is not available regarding its toxicity on testicular interstitium that has led this research. **Aims & Objectives:** Aim of the study was the evaluation of the toxicity of ribavirin in different doses on the testicular interstitium at different time points in albino rats. **Place and duration of study:** The experiment was performed in Anatomy Department of Postgraduate Medical Institute, Lahore and completed in three months. **Material & Methods:** 72 adult male albino rats were divided into 4 equal groups with 18 rats in every group. 0.75ml distilled water was given to Control (A) group intraperitoneally and Ribavirin was administered by same route in doses: 20mg, 100mg and 200mg/kg body weight, single dose for 5 days to experimental groups B, C and D respectively. Every group was subdivided into 3 small subgroups in accordance with 20th, 40th and 60th sacrificial days from the last dose of drug. On each sacrificial time 6 rats from a group were selected and sacrificed. Data was entered on SPSS 23.0 software and comparison among groups was made by Pearson's chi square test. P-value $\leq$0.05 was considered significant. **Results:** Interstitial widening and edema with congested vessels were observed in all study groups on 20th sacrificial day in comparison to control group. Signs of recovery in the form of decrease in widening, edema and congestion were only observed in rats of low dose groups on day 40th and 60th in comparison with high dose groups that didn't show reversibility of these changes. **Conclusion:** A patient who is taking Ribavirin as an antiviral therapy should be counselled about the reversibility of its gonadotoxicity.

Key words: Ribavirin, Testicular toxicity, Interstitium, Edema, Congestion.

INTRODUCTION

Hepatitis is a huge community health issue in the world which is linked with loss of many lives due to liver ailments.[1] Viruses, parasites, drugs, some toxic agents and certain autoimmune reactions against liver cells are the causative factors for hepatitis. Hepatitis C virus (HCV) detected in 1989 disseminates through contact with infected blood. 60 to 80 percent of long-standing infections bring about liver cirrhosis and hepatic cancer.[2] Ribavirin (RBV) orally and injectable Interferon alpha is an adjuvant therapy that has proved to be a powerful treatment for chronic Hepatitis C. RBV, an antiviral drug was produced in 1970 and its wide-ranging activity against viruses was spotlighted in 1972.[1] Its aerosol type is an effective remedy for Respiratory Syncytial Virus in youngsters and Intravenous type reduces mortality from Lassa and hemorrhagic fevers. RBV is considered as a purine (guanosine) nucleoside equivalent. Phosphorylation of RBV occurs inside the cell by host cell enzymes. RBV exists in three metabolic forms like Mono-, Di- and Triphosphates, they possess the ability to protect from many viruses. Ribavirin-5' –triphosphate is the main type intracellularly.[1,3,4] Ribavirin triphosphate ceases the duplication of various DNA and RNA viruses due to its broad-spectrum antiviral action. It may influence the formation of guanosine triphosphate; it can prevent the cap stage of viral messenger RNA or it may stop the viral RNA-dependent polymerase of certain viruses.[5]

Ribavirin is toxic to the embryos of laboratory animals. Patients (male and female) using this drug should not try for a baby during the treatment and for at least 6 months thereafter.[3,4,5] Intraperitoneal injection of RBV affects the testes after it gets absorbed from peritoneal cavity. It reaches the germ cells and exerts its mutagenicity. It prevents the action of Inosine monophosphate dehydrogenase that probably decreases guanosine triphosphate (GTP) concentration inside the cells. In this way, RBV is mutagenic for many viruses.[6]

Ribavirin is documented for the production of structural and functional disturbances in the tissues like bone marrow, liver, epididymis and testis of various experimental animals.[7,8,9,10]

Ribavirin seems to play its cytotoxic role by causing cell death due to blockage of cell division.[11]Its Metabolic by products proved genotoxic transiently even at prescribed doses for Crimean-Congo hemorrhagic fever.[12]

RBV is not suitable for either sex during six months prior to conception and its use is also prohibited during pregnancy due to its teratogenicity. Pregnancy registry of RBV was initiated in 2003 for documentation of its most probable embryotoxicity.[13] Semen aberrations were noted in patients with long standing infection of hepatitis C, taking adjunctive medication containing Pegylated interferons and Ribavirin.[14]

The cytotoxicity of RBV has been noted in seminiferous tubules and sperms of testes in previous studies but there is not enough evidence available that can highlight the effects of this drug on testicular interstitium. Therefore, this current research was undertaken to observe the effects of Ribavirin on the testicular interstitium of rats and reversibility of these changes was noted at various time points after discontinuation of drug.

MATERIAL AND METHODS

This study was conducted at Postgraduate Medical Institute, Lahore, approved by Review Board of University of Health Sciences, Lahore and it was a randomized controlled experimental research. 72 adult male albino rats weighing in the range of 180 - 200gms were purchased from National Institute of Health, Islamabad. The temperature of 24±2˚C and 12hrs light and dark cycle was maintained for rats. All animals were fed normal diet and were given water ad libitum. After adaptation of a week, rats were segregated into 4 groups each containing 18 rats by using table of random numbers. Ribavirin of Getz Pharma company, Karachi, Pakistan was used. Ribavirin was weighed on a laboratory scale in PGMI, Lahore.

Control group A: Rats were treated with 0.75ml/kg body weight (b.w) of distilled water by intraperitoneal injection once daily at 24 hrs. interval for 5 days.

Subgroups according to schedule of sacrifice:

A1, 20th day A2, 40th day A3, 60th day

Experimental group B: Ribavirin 20mg/kg b.w dissolved in 0.75ml distilled water was given by intraperitoneal injection once daily at 24hrs interval for 5 days.

Subgroups according to schedule of sacrifice:

B1, 20th day B2, 40th day, B3, 60th day

Experimental group C: Ribavirin 100mg/kg b.w dissolved in 0.75ml distilled water was given by intraperitoneal injection once daily at 24hrs interval for 5 days.

Subgroups according to schedule of sacrifice:

C1, 20th day C2, 40th day C3, 60th day

Experimental group D: Ribavirin 200mg/kg b.w dissolved in 0.75ml distilled water was given by intraperitoneal injection once daily at 24hrs interval for 5 days.

Subgroups according to schedule of sacrifice:

D1, 20th day D2, 40th day D3, 60th day

3 sacrificial days 20th, 40th and 60th from the last dose were selected and 3 subgroups of every group were made in accordance with these sacrificial times. In this way 12 subgroups in total were formed. On each sacrificial day 6 rats from every study group were chosen in an arbitrary manner. Their testes were dissected out after giving them anesthesia and for 18hrs testes were kept in Bouin's solution. Paraffin blocks were prepared after processing. Serial sections of 5 micrometer thickness were taken. Hematoxylin and Eosin stained testicular interstitium was observed under light microscope for certain changes, like changes in Leydig cells, changes in interstitial blood vessels and presence of cellular infiltration. These all were studied in comparison with controls using objective power 10x and 20x and findings were entered in tables 1, 2 and 3 respectively.

Statistical analysis:

Data was analyzed by SPSS, version 23. Data for qualitative variables was described by using frequency and percentage of each group. Comparison among groups was performed by Chi-square test taking P-value ≤ 0.05 as significant.

RESULTS

Interstitium contained loose connective tissue with blood vessels and Leydig cells. Leydig cells were rounded or polygonal cells with central nuclei. (Fig-1,2&3). On 20th sacrificial day from the last dose, B1 study group showed widening due to edema fluid accumulation in the interstitium. Blood vessels were dilated and filled with RBCs (Fig-1). C1 and D1 study groups showed more marked widening due to edema fluid accumulation in the interstitium. Vessels were dilated and filled with RBCs but Leydig cells did not show any structural change in all these groups (Fig-1). On the 40th sacrificial day from the last treatment, interstitium was showed

decrease in widening as well as in edema fluid content in the study group B2. Blood vessels were dilated and filled with RBCs. (Fig-2). C2 and D2 experimental groups still showed marked widening due to edema fluid in interstitium. Blood vessels were dilated and filled with RBCs. Leydig cells showed no structural change in any of these groups (Fig-2). When study groups were observed on 60th sacrificial day, B3 group showed nearly normal interstitium without any interstitial widening and edema fluid accumulation. Few blood vessels were still dilated and filled with RBCs (Fig-3). In C3 and D3 experimental groups, interstitium was still widened and edematous with dilated vessels containing RBCs. But Leydig cells were showing no structural change in these groups (Fig-3). All these changes in the interstitium including, accumulation of edema fluid and vascular congestion were noted for all study groups and a comparison was made with control groups at different sacrificial times by using Chi square test. The test results proved to be highly significant for all variables in all groups at various time points (Table-1, 2 & 3).

When control groups were compared with experimental groups at all time points, it was observed that the shape of the nucleus of Leydig cell was more or less same whether rounded or oval. Chromatin material distribution within the nucleus as well as number of nucleoli was also the same. With any dose level at any time point, no inflammatory cellular infiltration was observed in the interstitium of testes, no change in architecture as well as in the number of Leydig cells was noticed. The number was constant in the control and experimental groups at all time points even when grid method was applied.

Parameters	A1	B1	C1	D1	df	P-value
	n(%)	n(%)	n(%)	n(%)		
Interstitial edema	00(0.0)	6(100)	6(100)	6(100)	3	0.000**
Vascular congestion	00(0.0)	6(100)	6(100)	6(100)	3	0.000**

Table-1: Comparative relationship among study groups for histopathological changes in the interstitium on 20th sacrificial day

Parameters	A2	B2	C2	D2	df	P-value
	n(%)	n(%)	n(%)	n (%)		
Interstitial edema	00(0.0)	6(100)	6(100)	6(100)	3	0.000**
Vascular congestion	00(0.0)	6(100)	6(100)	6(100)	3	0.000**

Table-2: Comparative relationship among study groups for histopathological changes in the interstitium on 40th sacrificial day

Parameters	A3	B3	C3	D3	df	P-value
	n(%)	n(%)	n(%)	n(%)		
Interstitial edema	6(100)	00(0.0)	6(100)	6(100)	3	0.000**
Vascular congestion	00(0.0)	6(100)	6(100)	6(100)	3	0.000**

**P-value < 0.005 highly significant

Table-3: Comparative relationship among study groups for histopathological changes in the interstitium on 60thsacrificial day

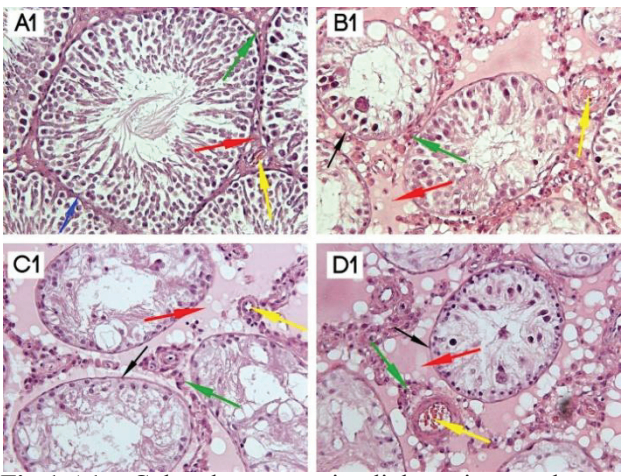

Fig-1:A1: Colored arrows in light micrograph are showing seminiferous tubules with germinal epithelium (Blue), Interstitium (Red) with Leydig cells (Green) and blood vessels (Yellow). (H&E, 20x)
B1,C1,D1: Colored arrows are showing shrunken seminiferous tubules with degenerating germ cells (Black), Interstitium widened due to edema fluid (Red), dilated blood vessels congested with RBCs (Yellow) and Clumps of Leydig cells (Green). (H&E, 20x)

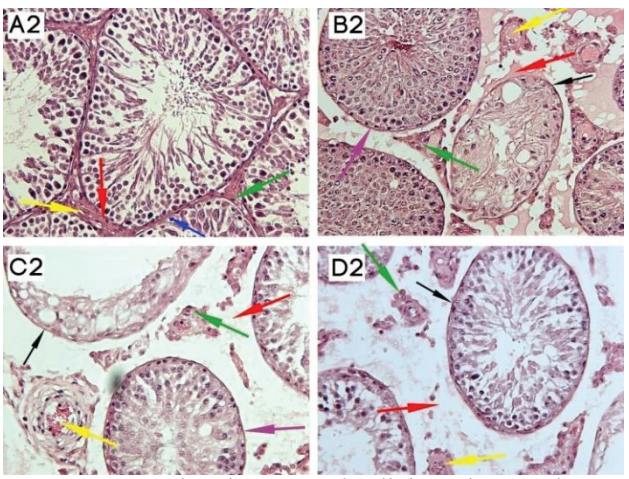

Fig-2: A2: Colored arrows in light micrograph are showing seminiferous tubules with germinal epithelium (Blue), Interstitium (Red) with Leydig cells (Green) and blood vessels (Yellow). (H&E, 20x)
B2,C2,D2: Colored arrows are showing shrunken seminiferous tubules with degenerating germ cells (Black), Seminiferous tubules with regenerating germ cells (Magenta), Interstitium widened due to edema fluid (Red), dilated blood vessels congested with RBCs (Yellow) and Clumps of Leydig cells (Green).(H&E, 20x)

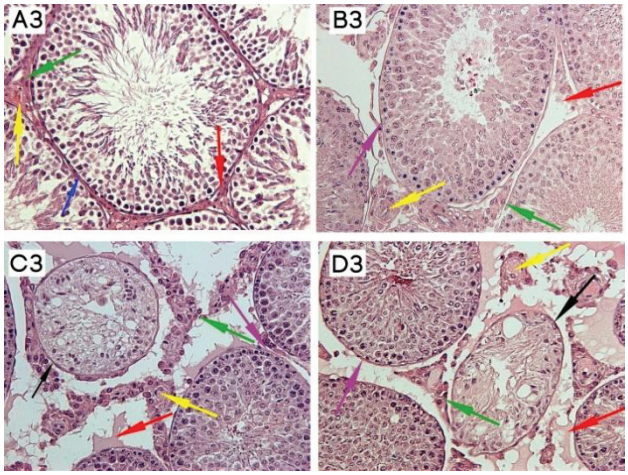

Fig-3: A3: Colored arrows in light micrograph are showing seminiferous tubules with germinal epithelium (Blue), Interstitium (Red), Blood vessels (Yellow), Leydig cells (Green). (H&E, 20x)

B3: Colored arrows are showing seminiferous tubules with regenerated germ cells (Magenta), Interstitium with no edema and widening (Red), Blood vessels (Yellow) and Leydig cells (Green). (H&E, 20x)

C3, D3: Colored arrows are showing shrunken seminiferous tubules with degenerated germ cells (Black), Seminiferous tubules with regenerated germ cells (Magenta), Interstitium widened due to edema fluid (Red), dilated blood vessels congested with RBCs (Yellow) and Clumps of Leydig cells (Green). (H&E, 20x)

DISCUSSION

In this study, after exposure to Ribavirin the examination of testicular interstitium revealed that there is widening due to edema, dilated and congested vessels were present in all experimental groups, and these changes in interstitium and vessels were found statistically significant. Neither any cellular infiltration, nor any structural change in Leydig cells was seen in testicular interstitium. It was observed that these interstitial changes were very marked on the 20th day of sacrifice. Gradual reduction in these changes was seen only in study group B2 on the 40th day. Reversal of these changes was seen only in group B3 on 60th day from the last dose of drug that might be due to decrease in toxicity of ribavirin with the passage of time but persisted in higher dose groups. The findings of this experiment are supported by one previously available research work on testes of experimental animals i.e, Autifi et al.,[1] (2017) observed widening of testicular interstitium and increase in Leydig cells after ribavirin administration to adult albino rats that was more in high dose groups but these changes reversed in low dose groups way before high dose groups when the drug's effects weaned off. In our study, no change was found in Leydig cells either in number or in structure. Narayana et al.,[15] (2005) found that ribavirin in different doses in rats causes degenerative changes in seminiferous tubules and decrease in Leydig cell number. Seminiferous tubules regenerate within 105 days after drug cessation due to its reversible cytotoxicity to rat's testes but normal Leydig cell number was not attained even after this period due to its endocrine disruption properties. No changes were found in Leydig cells in our study.

Watanabe et al.,[16] (2011) reported that Ribavirin (RBV) might have caused arterial and venous obstruction that resulted in vascular congestion and edema. Ribavirin caused vascular dilatation and congestion in interstitium. There may be leakage of fluid from these vessels that resulted in edema. It caused widening that slowly settled with the passage of time in low dose group B3 due to diminished toxic effects of this drug.[17]

Regarding edema, a prior study highlighted that Ribavirin caused transient pulmonary edema that cured when the drug was abandoned.[18] Pulmonary edema actually develops because of high capillary permeability due to damage of small vessels like capillaries of alveolar septas exposed to medications.[19] Ribavirin can induce injuries of vessels,[20] so edema may be a result of injury to vascular endothelium primarily or damage to alveolar epithelium (with secondary injury to small vessels). This can cause oozing of fluid and proteins into interstitial spaces and into the alveoli. [19] Edema is denoted by increased fluid in the interstitial spaces. The movement of fluid between vessels and interstitium is mainly due to the impact of inverse relationship between oncotic pressure and pressure exerted by fluid. Normally there is a balance between fluid outpouring and entry at the arteriolar and venular ends of capillaries respectively into the interstitium. When there is increase in capillary pressure or decrease in colloidal osmotic pressure it can result in more interstitial fluid. Locally hydrostatic pressure may increase due to reduced venous outpouring.[17] Ribavirin might have caused arterial occlusion or venous outflow obstruction according to Watanabe et al., (2011).[16] So it may have caused the above-mentioned pathology. Hirai et al.,[21] (2017) reported that testes need plenty of blood flow for spermatogenesis and testosterone production. Blood flow disturbance to the testes, either in the form of venous or arterial occlusion both cause problems in spermatogenesis due to oxidative stress. The venous blockage more commonly disturbs the organs like gonads which have only one venous efflux channel.[22]

Interstitial edema might be due to change in capillary permeability of the tissue. Narayana et al.,[15] (2005) noted that whenever seminiferous tubular epithelium deteriorated, there was a fall in testosterone levels that caused increase in Luteinizing hormone LH from anterior pituitary. The degeneration of the germ cell population of the testes is associated with changes in the pituitary histology and increase in the gonadotrophin component of the gland. Widmark et al.,[23] (1989) reported that high levels of LH may cause change in the capillary permeability and in this way, it can affect testicular microcirculation. Increased levels of LH might have caused increased capillary permeability and edema.

De Franceschi et al.,[24] (2000) reported that in patients of chronic hepatitis C, RBV causes hemolysis of RBCs due to membrane oxidative injury. Phillips and Henderson,[25] (2018) found that increased level of lactate dehydrogenase LDH confirms the diagnosis of hemolytic anemia as being one of its markers. The results of a study by Teng et al.,[26] (2006) showed that serum LDH can be an indicator of vessel formation(angiogenesis) in Acute myeloid leukemia AML in bone marrow. According to Mitchell.,[27] (2010) Angiogenesis is an important factor in the pathophysiology of edema. Vascular congestion and edema usually occur at the same time owing to the fact that congestion of capillaries may cause edema because of more leakage of fluid. In long standing over fullness of vessels due to decreased outflow, the stagnant deoxygenated blood leads to hypoxia. This can cause parenchymal cell degeneration with formation of scar tissue as seen in the form of seminiferous tubular epithelial deterioration.

CONCLUSION

Administration of Ribavirin, rats produced reversible toxic effects on the testicular interstitium. Low dose group was the only one that exhibited more recovery as compared to high dose groups which revealed less healing finally. Effect of toxicity on fertility of a patient must be kept in mind while prescribing this medicine. Patient's counselling who is taking this medicine is necessary regarding reversibility of its gonadotoxicity and effective contraceptive usage during the treatment.

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The Authors:

Dr. Alvia Batool
Associate Professor,
Department of Anatomy,
FMH College of Medicine & Dentistry, Lahore.

Dr. Maryam Fatima
House Officer,
Department of Neurology,
FMH College of Medicine & Dentistry, Lahore.

Prof. Fozia Farzana
Principal,
Department of Anatomy,
Continental Medical College, Lahore.

Corresponding Author:

Dr. Alvia Batool
Associate Professor,
Department of Anatomy,
FMH College of Medicine & Dentistry, Lahore.
E-mail: alvia.batool@gmail.com