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Evaluation of testicular cytotoxicity and genotoxicity of sofosbuvir and sofosbuvir - ribavirin in the adult male albino rats

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SUMMARY

Hepatitis C is a widely distributed problem all over the world, especially Egypt. Chronically infected people develop serious liver disease and now it is the most common cause for liver transplantation. Recently, a new regimen, sofosbuvir (sovaldi), alone or with combinations as sovaldi-ribavirin, was approved for treating this disease. There are limited studies that explore the effects of these drugs on the reproductive organs, and hence affection of male fertility while using these drugs. This study aims to throw more light on whether sovaldi or sovaldi-ribavirin causes testicular damaging effects in the adult male albino rats. We investigated the effect of this regimen in a dose equivalent to that used in the human (41 mg/kg once daily orally for sovaldi and 41 mg/kg twice daily orally for ribavirin) for consecutive 5 and 10 days. There was highly significant decrease in testosterone hormone level and marked degenerative changes in the seminiferous tubules and the testicular interstitium, with increase in collagen deposits in sovaldi treated rats, and in a more extensive manner in sovaldi-ribavirin treated rats. There was a significant increase of deoxyribonucleic acid (DNA) fragmentation in the treated groups after 10 days. However, there was a non-significant difference in DNA fragmentation in the treated groups after 5 days when compared with control. Immunohistochemistry detection of caspase-3 showed significant increase in its expression in the treated groups after either 5 or 10 days. This denoted the specificity of caspase-3 immunohistochemistry technique in the detection of early apoptotic changes. It was concluded that sovaldi and sovaldi-ribavirin induced gonadotoxic effects through induction of DNA fragmentation via upregulation of caspase-3, and that the resulting damaging effects increased with longer duration of drug intake.

Key words: Hepatitis C – Sovaldi – Sovaldi-ribavirin – Testis – Apoptosis

INTRODUCTION

One of the most prevalent leading causes of morbidity and even mortality all over the world is hepatitis C virus (HCV) with the highest prevalence in Egypt (Gomaa et al., 2017). Until now, there is no vaccine for HCV infection in spite of the existence of several routes of transmission (Reker and Islam, 2014).

Egypt, the single country with highest incidence of HCV infection in the world, has embarked on a government-sponsored mass treatment program using several combinations of directly acting antiviral drugs (DAAs) (El-Fishawy et al., 2016).

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Integration of pegylated interferon (PEGIFN) and ribavirin was prescribed by the standard care for chronic HCV infection between 2001 and 2011. In May 2011, two first-generation NS3/4A protease inhibitors, boceprevir, and telaprevir were approved in combination with PEG-IFN and ribavirin. In December 2013, a second-generation NS3/4A protease inhibitor, simeprevir was approved for use with PEG-IFN and/or ribavirin. One of the most used combinations is sovaldi-ribavirin (Yau and Yoshida, 2014).

Until recently, HCV infections were treated with two antiviral drugs that had to be taken for almost a year and produced serious side effects, and these treatments were successful only in a small fraction of people. In 2013, a new drug, sovaldi, was approved for treating HCV and is of special interest. This drug cures more than 90% of patients and is effective against several of the most common strains of HCV (Golanty and Edlin, 2015), with the chemical name L-Alanine, N-[[P(S),2′R]-2′-deoxy-2′-fluoro-2′-methyl-P-phenyl-5′-uridylyl]-, 1-methyl ethyl ester and a molecular formula of C22H29FN3O9P (Bhatia et al., 2014). NS5B is one of the non-structural proteins essential for viral ribonucleic acid (RNA) replication, and has been found to be a valuable target for DAAs (Lam et al., 2012). Sovaldi is a nucleotide analog that is a highly potent inhibitor of the NS5B polymerase in HCV. This drug has shown high efficacy in combination with several other drugs with and without PEG- INF, against HCV (Bhatia et al., 2014).

Sovaldi is a promising therapy for chronic HCV infection, as it offers several advantages over the existing therapies, particularly in dealing with patients with decompensated liver disease and patients who cannot tolerate interferon-containing therapies. On account of its excellent performance in clinical trials, this drug has got food drug administration (FDA) approval on 6 December, 2013, under the breakthrough therapy designation. This drug is effective against all HCV genotypes, has a better safety profile and low risk of development of resistance; however, careful clinical use and monitoring are still essential to gather more data on this drug (Bhatia et al., 2014).

Ribavirin is a non-selective, antihepatitis, antiviral drug. It was synthesized in 1970. The broad spectrum antiviral activity was reported in 1972 (Sidwell et al., 1972). In the early 1990s, ribavirin was studied for the treatment of HCV infection. Ribavirin had no significant effect on HCV RNA levels when used as a single agent, despite observations of improvements in serum aminotransferase levels (Di Bisceglie et al., 1995) and liver histology (Hoofnagle et al., 2003). Prolonging the course of treatment did not add any benefit in terms of virology clearance (Hoofnagle et al., 1996). Therefore, ribavirin has been used for the treatment of chronic HCV infection only in combination (Te et al., 2007).

Following the success of sovaldi or sovaldi-ribavirin regimen, further research is needed to clarify the optimal follow-up duration post-treatment and to evaluate the side effects of these drugs on different organs. Therefore, the testis was chosen in this study. Zhang et al. (2011) postulated that reproductive system is very sensitive to toxic chemicals because of the high multiplication rate of germ cells. On the other hand, the transmissible genetic damage from one generation to another takes place in this system only as reported by Au and Hsu (1980). Moreover, El-Atrebi et al. (2011) postulated that sexual dysfunction is a common side effect of Peg-IFN and ribavirin treatment, especially in middle-aged men in addition to advanced liver fibrosis that is an important co-factor in inducing sexual dysfunction during treatment.

The aim of this study was to evaluate the damaging effects of sovaldi and sovaldi- ribavirin at different durations on testicular tissue of adult male rats based on biochemical, histological, immunohistochemical and genetic assessments.

MATERIALS AND METHODS

Animals

Sixty adult male albino rats weighing 150-180 g were maintained in the animal house of the Faculty of Medicine, Menoufia University, Egypt. The rats were caged in standardized room conditions and allowed unlimited access to chow and water. The experiment started after one week of caging. All procedures involving the use of the rats were approved by the Animal Care and Use Committee, Faculty of Medicine, Menoufia University, Egypt.

Drugs

Sovaldi (Sofosbuvir, a product of Merck Company of pharmaceutical industries) was available in the form of tablets. Each tablet contained 400 mg of Sofosbuvir. They were crushed. The calculated dose was dissolved in distilled water.

Ribavirin (a product of Minapharm, Egypt) was available in the form of capsule. Each one contained 200 mg ribavirin. The contents of the capsule of the required dose were dissolved in distilled water.

Based on the clinical standard dose used for the human, animal equivalent dose (AED) calculation based on body surface area according to data adapted and modified from FDA draft guidelines was calculated. So, doses used in this study were calculated by using the following formula: - Rat Equivalent Dose (mg/kg) = Human does (mg/kg) × 6.2 (Nair and Jacop, 2016).

The clinical standard effective human dose for sovaldi is 400 mg once daily (Bhatia et al., 2014) and that for ribavirin is 800 mg/day divided into two equal doses (Jen et al., 2002) assuming the average human body weight 60Kg. So, the doses used in this study, 41 mg/kg once daily for sovaldi and

41 mg/kg twice daily for ribavirin, were approximately equivalent to the doses used in human.

Experimental design

The rats were divided into three groups (n=20 per group):

- Group I (control group): subdivided into two subgroups, (n= 10 per subgroup):-

Subgroup Ia: Ten rats received only standard diet and water for consecutive 5 days.

Subgroup Ib: Ten rats received only standard diet and water for consecutive 10 days.

- Group II (Sovaldi treated group): subdivided into two subgroups, (n= 10 per subgroup):-

Subgroup IIa:- Rats received sovaldi orally by gastric tube in a dose of 41 mg/kg dissolved in 2 ml distilled water once daily (equivalent to the clinical standard human dose 400 mg once daily) for 5 consecutive days.

Subgroup IIb:- Rats received sovaldi in the same previous dose and route of administration for 10 consecutive days.

- Group III (Sovaldi-Ribavirin treated group): subdivided into two subgroups, (n= 10 per subgroup):-

Subgroup IIIa:- Rats received sovaldi in the same previous dose and route of administration plus ribavirin orally by gastric tube in a dose of 41 mg/kg dissolved in 2 ml distilled water twice daily (approximately equivalent to the clinical standard human dose 400 mg twice/day) for 5 consecutive days.

Subgroup IIIb:- Rats received sovaldi and ribavirin in the same previous dose and route of administration for 10 consecutive days.

At the end of the experiment, the rats were anesthetized, intra-cardiac blood samples were collected, and the testes were dissected for further processing and examinations.

Biochemical analysis

Serum was obtained by centrifugation of blood samples. The concentration of testosterone hormone in blood was measured. Quantitative data were expressed in the form of mean ± standard deviation ($\bar{x}$ ± SD) and analyzed.

Molecular study

Fresh testicular tissues were subjected to molecular biological study as follows: Agarose gel technique for detection of DNA fragmentation as a result of apoptosis in testis.

Total genomic DNA extraction

Nucleic acid extraction was done according to the extraction method of Aljanabi and Martinez (1997), with some modifications introduced by El-Garawani and Hassab El-Nabi (2016), in which the direct staining of DNA sample was done. Apoptotic bands of DNA fragmentation appeared and located at 180 bp and its multiples 360, 540 and 720 bp against thirteen bands of 100 bp plus DNA ladder (Thermo Scientific™ O'Gene Ruler™, USA). The intensity of released DNA fragments was measured by image J software, as a mean of optical density values.

Histological, histochemical and immunohistochemical assessments

Testes were fixed in 10% neutral buffered formol solution for 24h and embedded in paraffin wax. For histological examination, 5-µm sections were deparaffinized and rehydrated using a graded ethanol (100%, 90%, and 70%) series and stained with hematoxylin and eosin (Hx&E) and Masson trichrome stain to show collagen fibers (Stevens and Wilson, 1996).

For caspase-3 immunohistochemistry, deparaffinized and hydrated testis sections were treated in 3% H2O2 for 5 min and rinsed with phosphate buffer saline (PBS) for 15 min. The sections were blocked with 1.5% normal goat serum in PBS and then incubated (45 min, room temperature) with rabbit polyclonal antihuman caspase-3 (0.5 µg/ml) in 1.5% normal goat serum in PBS. The sections then were incubated with biotin-conjugated goat antirabbit IgG (1:200, 1 h, room temperature), avidin-biotin-peroxidase complex (Santa Cruz Biotechnology, Inc., rabbit peroxidase kit; 1 h) and DAB solution. Sections were counterstained with hematoxylin. Positive reaction was visualized as brown coloration. Negative controls were done using the same steps, except that phosphate buffered saline was applied instead of the primary antibodies (Jackson et al., 2008).

Morphometrical study

The percentage surface area of collagen deposits and percentage of caspase-3 immunoreactive cells were measured. Measurement was done using an image analyzer (Image J program). From each slide of experimental groups, 9 fields were randomly selected.

The total field and histochemical stained areas were calculated and the percentage of caspase-3 immunoreactivity was calculated as follows: % immunoreactive stained cells = caspase-3 stained cell count/Total cell count × 100.

Statistical analysis

The data were collected, tabulated, and analyzed by SPSS (statistical package for social science) version 17.0 on IBM compatible computer (SPSS Inc., Chicago, IL, USA). The collected data were quantitative, which were described as mean ±SD and range; the data were compared using Mann Whitney U test. A P value of ≤ 0.05 was considered statistically significant. A P value of < 0.001 was considered statistically highly significant and P value > 0.05 was considered non-significant. This was done in the Public Health Department, Faculty of Medicine, Menoufia University, Egypt.

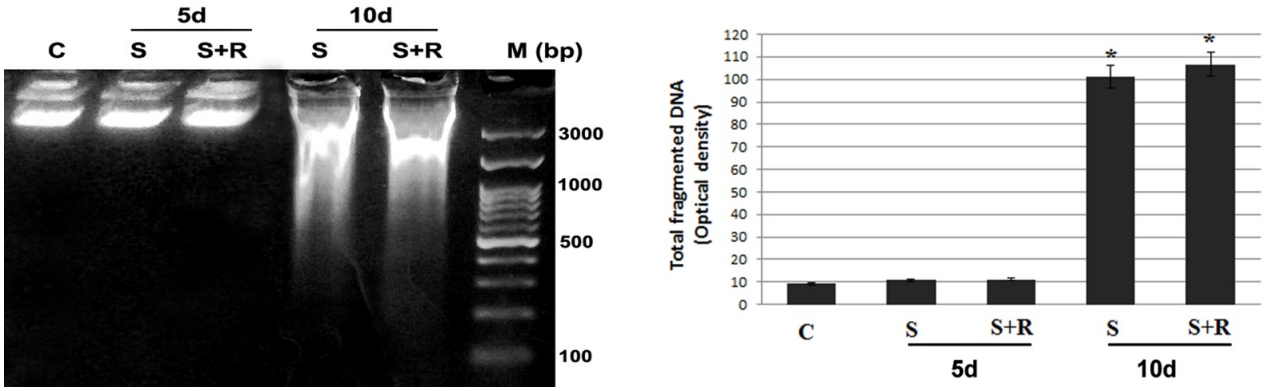

Fig 1. (a): Digital photograph of DNA electrophoresis of rat testis tissue showing the effect of sovaldi and sovaldi-ribavirin treated subgroups. Where C: control; S: sovaldi; S+R:sovaldi-ribavirin. 5d: after five days; 10d: after ten days and M: DNA marker. **(b)**: Total released DNA fragmentation in testis tissues of rats treated with sovaldi and sovaldi-ribavirin. C: control; S: sovaldi; S+R:sovaldi-ribavirin. 5d: after five days; 10d: after ten days. Data were presented as Mean± S.D., (n=6). Sovaldi and sovaldi-ribavirin treated rats after ten days showed significant increase of DNA fragmentation (P≤0.05) and after five days showed non-significant (P>0.05) with respect to control.

RESULTS

Endocrine disruptor effect of sovaldi and sovaldi-ribavirin in the rat testosterone hormone level

Analysis of testosterone hormone showed a non-significant difference (P>0.05) between the control subgroups after 5 & 10 days, while there was a significant decrease (P≤0.05) in its level in sovaldi and sovaldi-ribavirin treated rats after 5 and 10 days when compared with the corresponding control subgroups. Moreover, a significant decrease (P≤0.05) in testosterone hormone was observed in rats treated with sovaldi-ribavirin when compared with that treated with sovaldi only. In addition, there was a significant difference (P≤0.05) between the rats sacrificed after 5 days and that sacrificed after 10 days in the treated groups. (Table 1)

Total genomic DNA fragmentation and apoptosis effect of sovaldi and sovaldi-ribavirin treated rats

Optical density values of fragmented DNA extracted from testes of sovaldi treated rats and sovaldi-ribavirin treated rats after five days (10.9±2.29 and11±1.73) showed normal appearance of intact DNA that was non-significant (P>0.05) when compared with control (9.2±1.6). While the optical density values of fragmented DNA extracted from testes of sovaldi-treated rats and sovaldi-ribavirin treated rats after ten days (101.3±8.08 and 106.7±13.32 respectively) showed significant increase (P≤0.05) of DNA fragmentation appeared as smear pattern when compared with control (9.2±1.6) (Fig. 1 a,b).

Histopathological testicular changes of sovaldi and sovaldi-ribavirin-treated rats

- Hematoxylin and Eosin stain:

The testes of the control group either sacrificed 5 or 10 days after the beginning of the experiment

Table 1. Comparison between the studied groups regarding testosterone level

Subgroups	The studied groups			Test	P value
	Group I	**Group II**	**Group III**	**U test**	
Testosterone (ng/dl) in Subgroup (a) [n=5]				1.99	0.0471[1]
Mean±SD	1.93±0.39	1.37±0.37	0.92±0.24	2.61	0.0092[2]
Range	1.4 – 2.45	1.05 – 2.0	0.65 – 1.3	2.01	0.0453[3]
Testosterone (ng/dl) in Subgroup (b) [n=5]				2.41	0.0161[1]
Mean± SD	1.46±0.36	0.77±0.33	0.32±0.08	2.64	0.0082[2]
Range	1 – 1.9	0.35 – 1.1	0.2 – 0.40	2.21	0.0273[3]
U test	1.68	2.42	2.63		
P value	0.094[4]	0.024[4]	0.0094[4]		

Subgroup (a): measurement after 5 days

Subgroup (b): measurement after 10 days

U test = Mann Whitney U test

1 = comparing group I with group II

2 = comparing group I with group III

3 = comparing group II with group III

4 = comparing subgroup a and subgroup b in each group

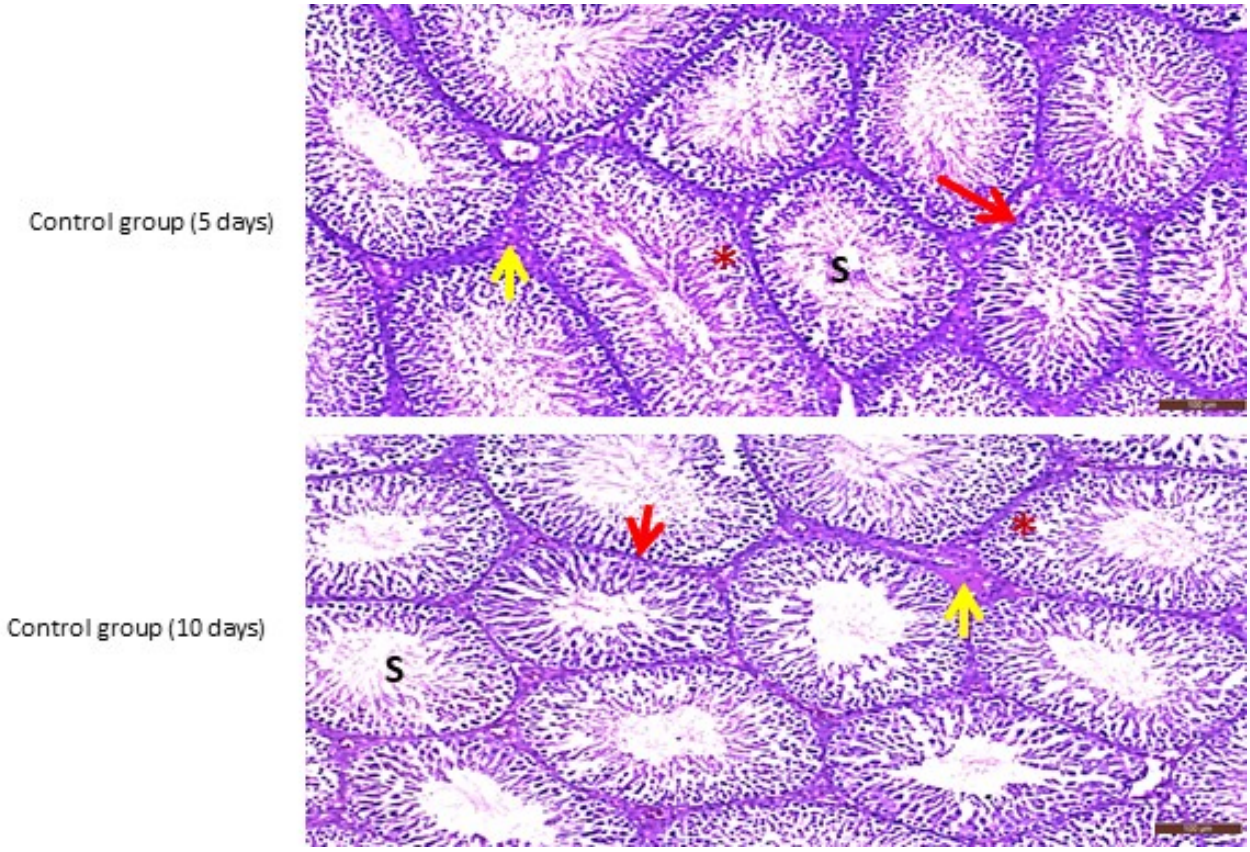

Fig 2. Representative micrographs of a testicular section of a control rat showing seminiferous tubules lined by spermatogonia (red arrow), primary spermatocytes (star) within the seminiferous tubules, and the tails of spermatozoa (S) are observed in the center of the seminiferous tubule. A few Leydig cells with an acidophilic cytoplasm (yellow arrow) are scattered between the seminiferous tubules (hematoxylin-eosin, 20x).

revealed normal testicular structure. The seminiferous tubules appeared uniform lined by regularly arranged spermatogenic cells at different stages of maturation. They were separated by interstitial tissue. The interstitial cells of Leydig appeared rounded or polygonal in shape, with acidophilic cytoplasm and large rounded nuclei. The shown spermatogenic cells were spermatogonia, primary spermatocytes, and spermatozoa filling the lumen of the tubules (Fig. 2).

Sovaldi treated group either sacrificed after 5 or 10 days showed distortion of the seminiferous tubules, especially in the rats sacrificed at the 10th day. The separation of spermatogenic cells from

Table 2. Comparison between the studied groups regarding % of surface area of collagen deposition.

Subgroups	The studied groups			Test	P value
	Group I	**Group II**	**Group III**	**U test**	
%surface area of collagen deposition in Subgroup (a)				2.61	0.009[1]
Mean± SD	6.44±1.63	12.26±3.09	19.16±2.51	2.61	0.009[2]
Range	4.5 – 8.4	9.3 – 17	16.2 – 22.9	2.40	0.016[3]
%surface area of collagen deposition in Subgroup (b)				2.61	0.009[1]
Mean± SD	7.28±1.55	18.3±2.07	24.12±3.16	2.61	0.009[2]
Range	5.6 – 9	15.6 – 21.3	20.8 – 27.9	2.40	0.016[3]
U test	0.94	2.40	2.19		
P value	0.35[4]	0.016[4]	0.028[4]		

Subgroup (a): measurement after 5 days

Subgroup (b): measurement after 10 days

U test = Mann Whitney U test

1 = comparing group I with group II

2 = comparing group I with group III

3 = comparing group II with group III

4 = comparing subgroup a and subgroup b in each group

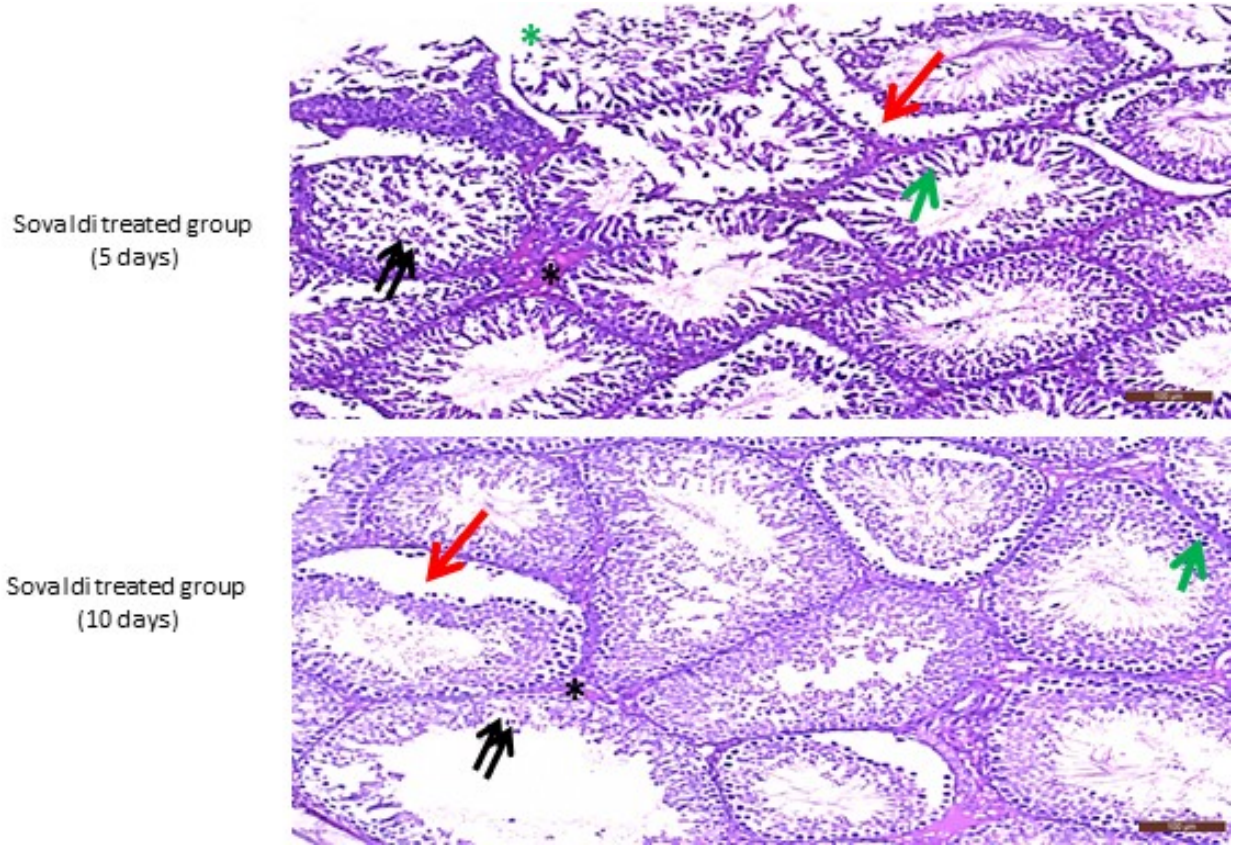

Fig 3. Representative micrographs of a testicular section of a sovaldi treated rat showing distortion of some seminiferous tubules with disorganization of the spermatogenic cells (double black arrows), cytoplasmic vacuolation (green arrow) of the spermatogenic cells, sloughing of the spermatogenic cells from the underlying basement membrane (red arrow) and atrophy of Leydig cells (asterisk). These changes are more obvious in the sovaldi treated group for 10 days (hematoxylin-eosin, 20x).

their underlying basement membrane was observed in most of seminiferous tubules. Moreover, there were marked cytoplasmic vacuolations of the spermatogenic cells and atrophy of Leydig cells (Fig. 3).

The histological changes that were seen in the testicular sections of the rats treated with both sovaldi and ribavirin were nearly similar to that occurred in sovaldi treated group, but in an extensive manner. There was a marked distortion of the testicular seminiferous tubules with presence of congested dilated blood vessels in the testicular in-

Table 3. Comparison between the studied groups regarding % of surface area of collagen deposition.

Subgroups	Studied groups			Test	P value
	Group I	Group II	Group III	U test	
% of spermatogenic cells in Subgroup (a) Mean± SD Range	10.4±2.30 8 – 13	60.60±4.83 55 – 66	72.60±6.80 64 – 80	2.91 2.61 2.19	0.009[1] 0.009[2] 0.028[3]
% of spermatogenic cells in Subgroup (b) Mean± SD Range	10.0±2.34 7 – 12	88.4±6.19 82 – 96	97.40±2.61 94 – 100	2.91 2.61 2.22	0.009[1] 0.009[2] 0.026[3]
U test	0.43	2.62	2.62		
P value	0.67[4]	0.009[4]	0.009[4]		

Subgroup (a): measurement after 5 days

Subgroup (b): measurement after 10 days

U test = Mann Whitney U test

1 = comparing group I with group II

2 = comparing group I with group III

3 = comparing group II with group III

4 = comparing subgroup a and subgroup b in each group

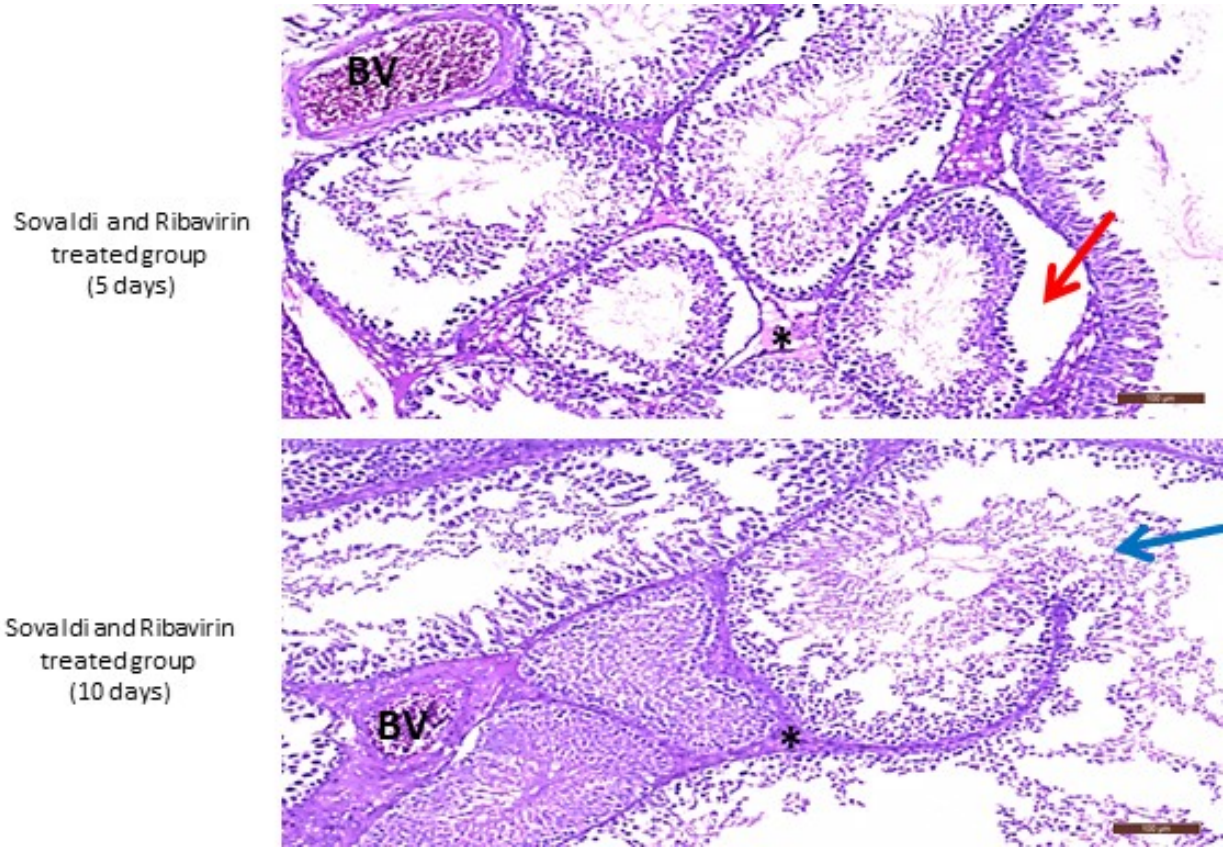

Fig 4. Representative micrographs of a testicular section of a sovaldi-ribavirin treated rat showing marked distortion of the seminiferous tubules with intense sloughing (red arrow) of the disorganized spermatogenic cells from the basement membrane, disruption of the basement membrane (blue arrow) specially 10 days after drugs intake with presence of dilated congested blood vessels (BV) and atrophy of Leydig cells (asterisk) (hematoxylin-eosin, 20x).

terstitium (Fig. 4).

- Masson trichrome stain:

There was a fine collagen deposition, especially peritubular and in the tunica albuginea in the control group that increased in the sovaldi treated group, and became more obvious in sovaldi-ribavirin treated group. Statistically, there was a significant increase in the % surface area of colla -gen deposits in sovaldi and sovaldi-ribavirin treated groups when compared to the control group. Moreover, a significant increase in collagen deposits was more in sovaldi-ribavirin treated rats

Table 4. Comparison between the studied groups regarding % of surface area of collagen deposition.

Subgroups	Studied groups			Test	P value
	Group I	**Group II**	**Group III**	**U test**	
Leydig cells in Subgroup (a) Mean± SD Range	5.2±1.92 3 – 8	43.6±6.11 38 – 52	59.2±5.26 53 – 66	2.61 2.61 2.56	0.009[1] 0.009[2] 0.01[3]
Leydig cells in Subgroup (b) Mean± SD Range	5.6± 1.81 4 – 8	85.2±5.72 80 – 93	95.4±4.50 90 – 102	2.63 2.62 2.31	0.009[1] 0.009[2] 0.02[3]
U test	0.32	2.62	2.61		
P value	0.75[4]	0.009[4]	0.009[4]		

Subgroup (a): measurement after 5 days

Subgroup (b): measurement after 10 days

U test = Mann Whitney U test

1 = comparing group I with group II

2 = comparing group I with group III

3 = comparing group II with group III

4 = comparing subgroup a and subgroup b in each group

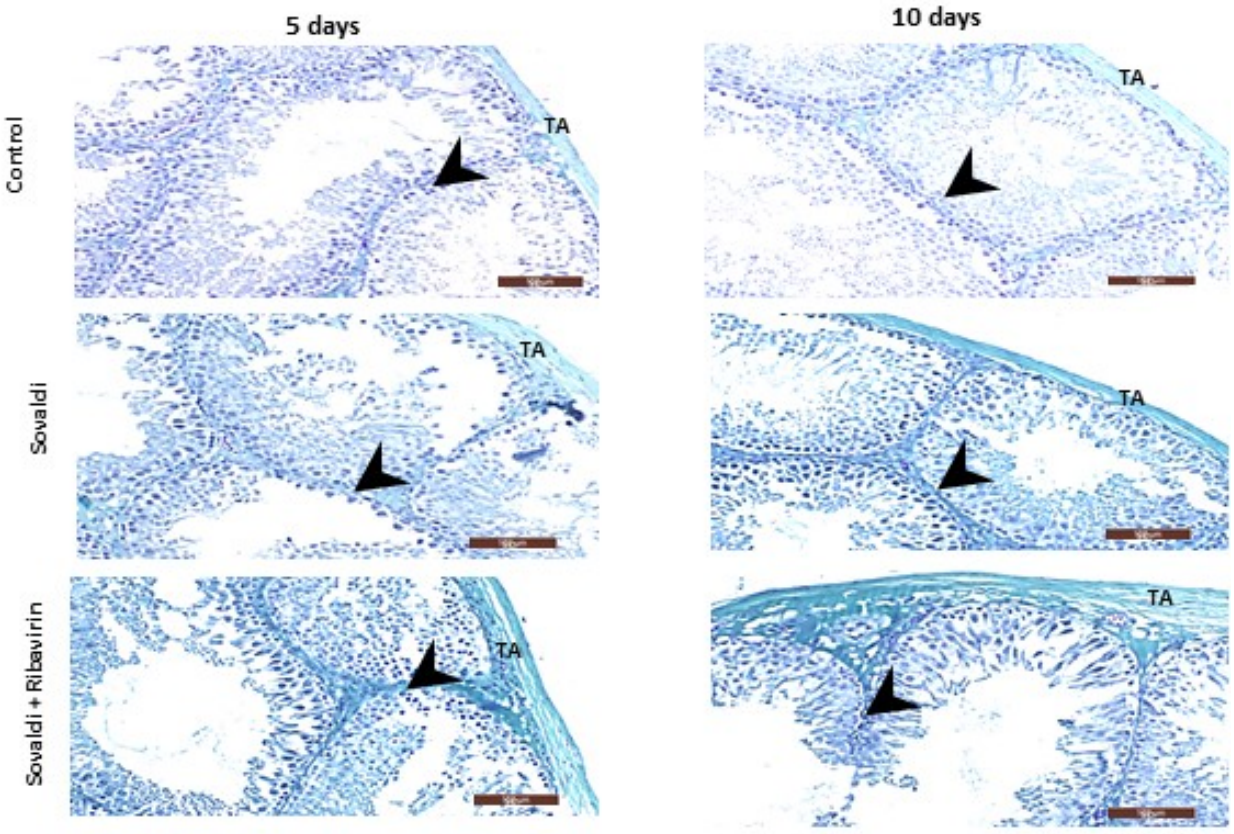

Fig 5. Representative micrographs of the testicular sections of the different experimental groups showing fine collagen deposits either in tunica albuginea (TA) or peritubular (arrow head) in the control group that increases in sovaldi treated group to be more dense in sovaldi-ribavirin treated group especially 10 days after drugs intake (Masson trichrome, 40x).

when compared with that of sovaldi treated group. In addition, the significant difference between rats treated for 10 days and those treated for only 5 days was noted (Fig. 5, Table 2).

Apoptosis effect in testis of sovaldi and sovaldi-ribavirin treated rats

Immunoexpression of caspase-3, a marker for apoptosis, revealed that there was a negative immunoexpression for caspase-3 in the control group, except for very few positive cells, either spermatogenic or Leydig cells. However, in the sovaldi treated group there was an increase in the number of positive cells especially 10 days after sovaldi intake. In sovaldi-ribavirin treated group, the immunoexpression to caspase-3 was more extensive especially with the increased duration. Statistically, there was a significant increase in the % of caspase-3 positive cells in sovaldi and sovaldi-ribavirin treated groups when compared with the control group. Furthermore, a significant increase was more in sovaldi-ribavirin treated rats when compared with that of sovaldi treated (Fig. 6, Tables 3, 4).

DISCUSSION

The therapeutic effects of sovaldi and sovaldi-ribavirin on Hepatitis C have been well documented. However, the results of our experimental study reveal that administration of a therapeutic dose of sovaldi and sovaldi-ribavirin promoted male reproductive toxicity in rats. Up to our knowledge, there were no documented researches on the effect of sovaldi and sovaldi-ribavirin on testicular human, with limited studies to explore the effects of these drugs on the rat reproductive organs and hence affection of male fertility while using these drugs.

These entailed the aim of this study.

Regarding the biochemical analysis of testosterone hormone, there was a significant decrease in the groups treated with sovaldi and sovaldi-ribavirin. Moreover, examination of the testicular sections of sovaldi treated rats either sacrificed 5 or 10 days after drug intake showed distortion of the seminiferous tubules, sloughing of the spermatogenic cells from their underlying basement membrane, appearance of vacuoles in the epithelium and atrophy of Leydig cells. There was an extensive distortion of the testicular seminiferous tubules with presence of congested dilated blood vessels in the testicular interstitium, and appearance of more gaps and vacuoles in testicular sections of the rats treated with both sovaldi and ribavirin. These findings are comparable to the study outcomes of Narayana et al. (2005), who confirmed that ribavirin induced formation of vacuoles and gaps in the seminiferous epithelium in all dose groups at

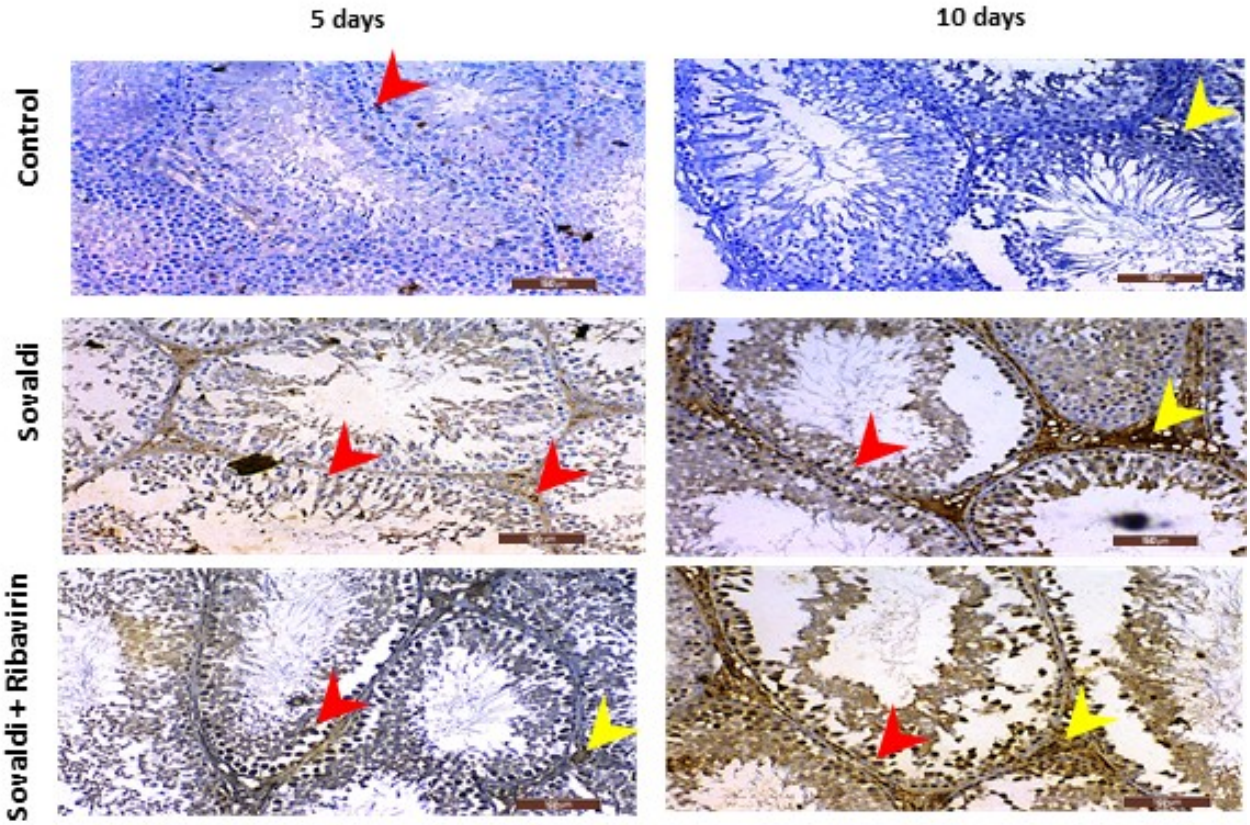

Fig 6. Representative micrographs of the testicular sections of the different experimental groups: negative immunoreaction for caspase-3 except for very few positive spermatogenic (red arrow head) and Leydig cells (yellow arrow head). Sovaldi-treated group sacrificed 5 & 10 days clarifying increase in the number of positive cells that is more obvious in testicular sections from sovaldi treated group for 10 days sovaldi-ribavirin treated group sacrificed 5 & 10 days showing extensive immunopositive expression (Immunoexpression for caspase-3, 40x).

all sacrifice times. The appearance of vacuoles in the seminiferous epithelium indicated that the drug caused damage to the Sertoli cell structure in terms of dilatation of the endoplasmic reticulum as considered by De Krester and Kerr, (1994). However, Chapin et al. (1984) had the opinion that the vacuoles indicated a non-specific injury to germ cells.. Kumar et al. (2006) added that epithelial sloughing was an indicator of Sertoli cells damage. In addition, Narayana et al. (2005) stated that the gaps in the seminiferous epithelium appeared as a result of the removal of germ cells by sloughing, which may be due to damage to intercellular bridges formed by the microtubules between the Sertoli cells and the germ cells.

Moreover, the biochemical and histological results in this work agreed with Ahtiainen et al. (2004), who postulated that reduction in serum testosterone level may be due to atrophy of Leydig cells. These changes were described by Benzoni et al. (2008) as testosterone supports spermatogenesis, sperm maturation and sexual function, thus any disruption in testosterone biosynthesis could adversely affect male fertility. The changes in the seminiferous tubules, as observed during histopathological examination, might be a result of hormonal effect and not consequence of a direct effect. In addition, this was confirmed by Narayana et al. (2005), who reported that ribavirin has some cytotoxic effects on Leydig cells, which resulted in a decrease in their number and stated that ribavirin has a moderate but prolonged effect on testosterone levels in the rat and the lowered level of the hormone was due to fewer Leydig cells.

From the previous results, it could be concluded that sovaldi and sovaldi-ribavirin function like an endocrine-disruptor in the rat.

Furthermore, in the present study, there was a significant increase in collagen deposits in the sovaldi treated subgroups and became more obvious in sovaldi-ribavirin treated subgroups. These findings were in correlation with Ahtiainen et al. (2004), who stated that increase in connective tissue between seminiferous tubules was a consequence of atrophy of tubules.

Molecular study of sovaldi and sovaldi-ribavirin treated rats in this work showed that significant increase ($P \leq 0.05$) of DNA fragmentation after ten days appeared as smear pattern when compared with control. Moreover, there was a significant increase ($P \leq 0.05$) in the percentage of caspase-3 positive cells, especially ten days after sovaldi intake. In addition, the immunoexpression of caspase-3 in sovaldi-ribavirin treated subgroups was more

extensive especially after ten days. These findings were in agreement with Lasheen et al. (2015), who hypothesized that the increase in testicular apoptosis was the result of decline of gonadotropins, and subsequently testosterone hormonal level that influenced the testicular cellular viability. Furthermore, El-Sharaky et al. (2010) reported that depletion of testosterone in the rats resulted in increased germ cell apoptosis. In addition, testosterone could affect Sertoli cells' function and germinal cell degeneration and dislocation could take place due to damage in function of Sertoli cells, and decreased testosterone level had been reported to enhance premature detachment of epithelial cells as mentioned by Kumar et al. (2006) and Najafi et al. (2010).

Additionally, our work showed that there was increase in the number of caspase-3 positive cells after 5 days in sovaldi and sovaldi-ribavirin treated groups. This may be due to the central importance of caspase proteinases, particularly caspase-3, as the principal mediators of apoptosis thus considered as the primary activator of apoptotic DNA fragmentation, as suggested by Wolf et al. (1999).

The detection of caspase-3 immunohistochemically was considered a specific method for detection of apoptosis as clarified by Arroyo et al. (2010); Cheng et al. (2011). Rainaldi et al. (2008) added that agarose gel electrophoresis, along with other methods, was successfully used to confirm apoptosis. Moreover, Duan et al. (2003) stated that detection of caspase-3 could be a more unique, direct and sensitive indicator of apoptosis than detection of secondary process as DNA fragmentation.

The findings of lowered testosterone hormone, testicular DNA fragmentation, caspase-3 activation and testicular damage, along with increased collagen deposits, were considered the most sensitive parameters for detection of testicular toxicity that could be of key importance for the clinical practice.

CONCLUSION

This study demonstrated the potential adverse effects of sovaldi and sovaldi-ribavirin on reproductive system and function in male rats, and showed that sovaldi and sovaldi-ribavirin induced reproductive disorders as revealed by reduction of serum testosterone level, toxic and degenerative effects on the histological architecture of testes, increase in collagen deposits, increased number of caspase -3 positive cells and DNA fragmentation. Also these reproductive disorders become more aggressive with increased duration of drugs intake.

Finally, sovaldi and sovaldi-ribavirin may induce reduction in the potential fertility of adult male rats due to their potential structural adverse effects on rats' testes, so the physicians, while prescribing these drugs to the patients, must consider their possible gonadotoxic effects on his fertility. Further studies are recommended to demonstrate whether these damaging effects will be reversible or not, and to use a protective agent to ameliorate the testicular structural changes induced by sovaldi and sovaldi-ribavirin.

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