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Hepatic histological alterations and biochemical changes induced by sildenafil overdoses

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Abstract: Sildenafil is used for the treatment of erectile dysfunction and is helping millions of men around the world to achieve and maintain a long lasting erection. Fifty healthy male rabbits (*Oryctolagus cuniculus*) were used in the present study and exposed daily to sildenafil (0, 1, 3, 6, 9mg/kg) for 5 days per week for 7 weeks to investigate the biochemical changes and alterations in the hepatic tissues induced by this drug overdosing. In comparison with respective control rabbits, sildenafil overdoses elevated significantly (p-value<0.05, ANOVA test) alanine aminotransferase (ALT), aspartate aminotransferase (AST), testosterone, follicular stimulating hormone and total protein, while creatinine and urea were lowered with no significant alteration was observed in uric acid and luteinizing hormone concentration. Also sildenafil provoked hepatocytes nuclear alterations, necrosis, hydropic degeneration, bile duct hyperplasia, Kupffer cells hyperplasia, inflammatory cells infiltration, hepatic vessels congestion and evident partial depletion of glycogen content. The results show that subchronic exposure to sildenafil overdoses exhibits significant biochemical and alterations in the hepatic tissues that might affect the functions of the liver and other vital organs.

Keywords: Liver, sildenafil, hydropic degeneration, overdoses, bile duct hyperplasia, Necrosis, AST, ALT.

INTRODUCTION

Sildenafil is used for the erectile dysfunction treatment and is being used by millions of people to maintain a long lasting erection (Bollel, 1996). This drug inhibits phosphodiestrase-5 causing nitric oxide release from the penile tissues, leading to the relaxation of corpus cavernosum smooth muscles, increasing inflow of blood into the spongy tissue of the penis and then causing an erection (Schultheiss *et al*., 1997). Sildenafil was reported to be supportive to men with erectile dysfunction, including those suffer from diabetes, hypertension, spinal cord injuries, multiple sclerosis, depression, schizophrenia and men after prostatectomy (Basu and Ryder, 2004; Feldman *et al*., 1999; Deforge *et al*., 2006; Nadipati *et al*., 2006; Fowler *et al*., 2005; Fava *et al*., 2006; Gopalkrishnan *et al*., 2006). Moreover, sildenafil exhibited an impact in children who suffered from pulmonary hypertension (Derchi *et al*., 2005; Huddleston *et al*., 2009). In addition, some reports have indicated that this drug can be used to tolerate benign prostatic hyperplasia (Wang, 2010). Also, some investigations showed that this drug attenuated renal injury especially in nephrotoxicity, increased morphine antinociception, elevated testosterone level and inhibited erythrocytes carbonic anhydrase activity (Karmoosh, 2002; Yoo *et al*., 2002; Lee *et al*., 2008; Yoon *et al*., 2008; Abdulkader *et al*., 2009; Saraiva *et al*., 2009; Sergeant *et al*., 2009). It was also reported that sildenafil altered the ultrastructure of Leydig cells, reduced pulmonary fibrosis and augmented histological alteration of the myocardial cells induced by hypertension or amlodipine (Sergeant *et al*., 2009; Ferreira-Melo *et al*., 2006; Aboutable *et al*., 2008; Hemnes *et al*., 2008; Oruc *et al*., 2010).

Sildenafil overdoses uptake is mainly seen among men suffer from erectile dysfunction where the stigma of this disease surrounds them and their partners. Clinically, this drug proved to be an effective drug in elderly men but its efficacy rate decreased with age increasing (Muller *et al*., 2007; Brown *et al*., 2009). Also, sildenafil popularity is increasing with young adults due to the belief that the drug increases libido, improves sexual performance and increases penis size. Research studies also indicate that sildenafil is so widely used by body builders and athletes and so far is legal in the world of sports (Spring *et al*., 2006). On the other hand, sildenafil overdoses fatality is on the rise and have reached crisis levels in certain countries (Wada *et al*., 2009).

Case studies showed that sildenafil overdose may result in facial flushing, hearing impairment, nose bleeding, nose stuffiness, hypotension, chest pain, priapism, tachycardia and arrhythmia (Krenzelok, 2000; Hicklin *et al*., 2000; Pagani *et al*., 2005; Wills *et al*., 2007; Maddox *et al*., 2009). In addition, sildenafil overdose was reported to cause death among men with potential arrhythmia (Tracqui *et al*., 2002). Exposure of male rabbits to overdoses of sildenafil had provoked tubular and interstitial testicular histological alterations including spermatocytes karyopyknosis, spermatocytes degeneration, and arrest of spermatogenesis (Jarrar, 2011). Also, sildenafil high doses caused cellular degenerative changes

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and intercellular vacuolation in the stroma of the medial geniculate body (Kurt *et al*., 2004).

The hepatic tissues alterations and biochemical characterization induced by sildenafil overdosing intoxication is not yet well identified. The present work aims to characterize the possible alterations in the hepatic tissues and biochemical changes following experimental sildenafil overdoses.

MATERIALS AND METHODS

Experimental animals

A total of 50 adult healthy male rabbits (*Oryctolagus cuniculus*) of similar age with a weight of 1050-1100gm were used throughout the present study. All rabbits were obtained from the animal house of the Medical Laboratory Sciences Department, Aljouf University and were randomly divided into 5 groups (assigned to control group and four test groups) of 10 animals each. The animals were housed at room temperature (24±1°C) and provided with commercial pellets and tap water *ad libitum*.

Drugs and chemicals

Sildenafil in the form of tablets containing sildenafil citrate equivalent to 50 or 100mg of sildenafil (Fluka, Switzerland), were utilized in the present work.

Experimental protocol

All members of sildenafil treated groups were exposed to intraperitoneal (i.p.) injection with a daily single dose of (0,1, 3, 6, 9mg/kg/bw respectively) for 5 days per week for 7 weeks. The selection of these doses and the route of administration was based on data from previous works (Kurt *et al*., 2004; Shafiei *et al*., 2006). The drug solution was prepared in sterile saline (0.45%, pH=4.35-4.5) at 37 $^{\circ}$C immediately before administration to the rabbits (Wang *et al.* 2008). Drug solutions were prepared so that the needed dose could be administered in one ml volume. Each control rabbit was exposed to a daily single i.p. injection of one ml of sterile sodium chloride (0.45%).

Sample preparation

Rabbits were weighed at the beginning of treatment and on days of euthanization where two rabbits from each group were euthanized on days 14, 28 and 35 following sildenafil administration for histological examination. Blood sample was collected from each rabbit of all groups on day 35 of the experiment. Liver was taken from each euthanized rabbits and the percentage absolute liver weight was determined while the grade of change in the liver index (L_x) induced by sildenafil overdoses was calculated according to the following equation:

L_x =Average weight of the experimental livers/ Average weight of the experimental animals

Weight of the control livers/weight of the control animals

Biochemical analysis

Serum samples were separated by centrifugation and analyzed for the following biochemical parameters: AST, ALT, uric acid, urea, total protein, luteinizing hormone, follicular stimulating hormone and testosterone.

Histological examination

Fresh liver biopsies were cut out from the median lobe of each rabbit of all groups and were used for histological processing included fixation in 10% neutral buffered formalin, dehydration with ethanol, clearing with chloroform, wax impregnation with melted paraffin wax, embedding and sectioning. Tissue processing was carried out by automatic Tissue Processor (Thermo Shandon Company). Paraffin sections (4-5µm) from all experimental rabbits of all groups were applied for the following conventional histological and histochemical stains: hematoxylin and eosin (H&E), Best's carmine stain, periodic acid-Schiff (PAS) method, Mallory trichrome and Perl's reaction (Pearse, 1985; Bancroft and Stevens, 1986; Kiernan, 1989; Jarrar and Taib, 2008). Stained sections of control and treated rabbits were examined and viewed for histological and histochemical alterations in the hepatic tissues.

STATISTICAL ANALYSIS

All continuous results in this study were expressed by the average ± sd. One-way ANOVA test (p-value<0.05) was used as a statistical tool for comparison the effect of different sildenafil doses. A p-value<0.05 was used to reject the null hypothesis.

The experimental protocol of the present work was approved by the local Bioethics Committee of Aljouf University. The international guidelines for care and use of laboratory animals were followed in the experimental work of the present study.

RESULTS

The week variation of the body weight during sildenafil exposure is given in table 1 while the liver weights, the percentage absolute liver weight to control one and the grade of change in the liver index are seen in table 2. After 5 weeks of sildenafil administration, no significant change in mean body weight was observed in rabbits received 3 or 6mg/kg/bw of sildenafil from the control ones. However, 9mg/kg/body weight of sildenafil showed a significant (p-value <0.05) reduction in the rabbits body weight which was associated with the time exposure to the drug. Sildenafil treatment (1-6mg/kg/bw) showed a week dose-dependence reduction in the percentage absolute weight of the liver in the rabbits under study, which failed to reach the statistical significance (p-value <0.05, ANOVA test).

Biochemical changes

Compared with the control serum, sildenafil overdoses elevated significantly (p-value <0.05, ANOVA), AST, ALT, total protein, testosterone and follicular stimulating hormone while creatinine and urea, were lowered with no significant alteration was observed in uric acid and luteinizing hormone concentration (table 3).

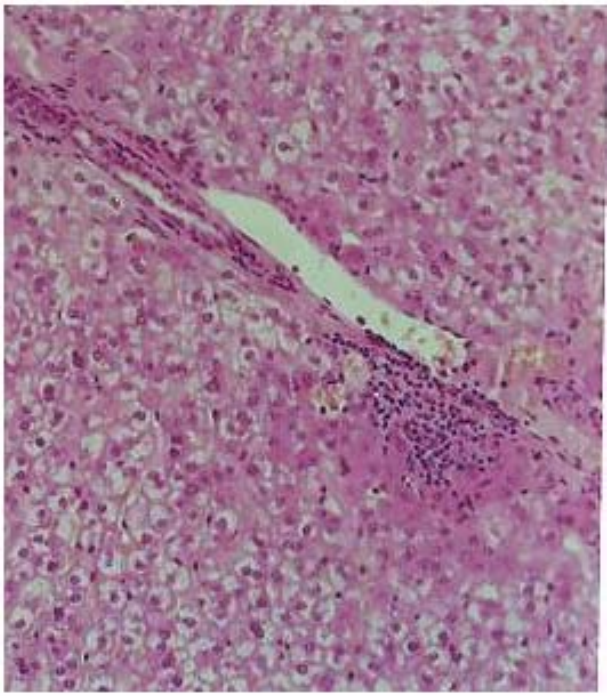

Fig. 1: Sildenafil-treated rabbit received 9mg/kg for 35 days demonstrating anisonucleosis, binucleation, pyknosis, karyorrhexis and karylysis. H&E stain.

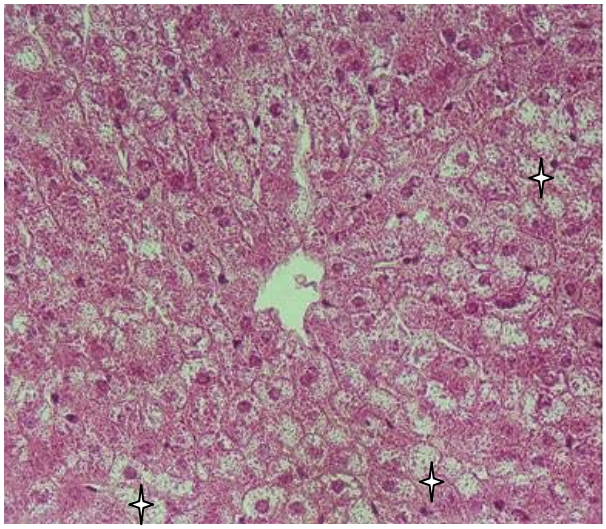

Fig. 2: Sildenafil-treated rabbit received 9mg/kg for 35 days demonstrating necrosis (stars). H&E stain.

Histological alterations

The liver lobular architecture was preserved and kept intact in all treated rabbits even after 35 days of drug administration while the lobular zonation accentuation was not affected due to sildenafil overdoses. The following histological and histochemical alterations were detected in the hepatic tissues of rabbits exposed to sildenafil:

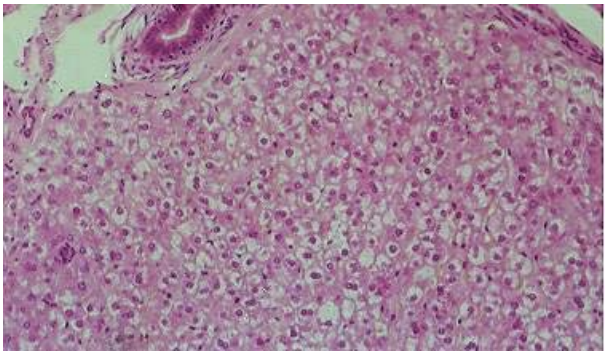

Fig. 3: Sildenafil-treated rabbit received 6mg/kg for 28 days demonstrating hydropic degeneration.

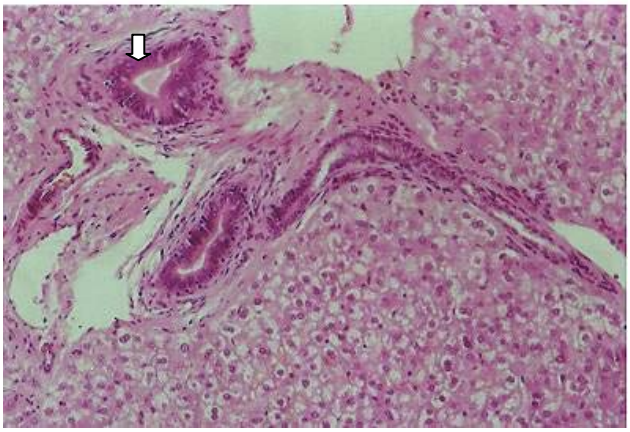

Fig. 4: Sildenafil-treated rabbit received 6mg/kg for 28 days demonstrating bile duct hyperplasia (arrow). H&E stain.

Nuclear alterations

Nuclear vesiculation, anisonucleosis, marked binucleation, karyopyknosis, karyorrhexis and karyolysis were observed in the hepatocytes of rabbits exposed to sildenafil. Nuclear vesiculation appeared early in the hepatocytes of these rabbits but become less frequent after 28 days and more (fig. 1). Anisonucleosis, binucleation, karyorrhexis and karyolysis were seen in the hepatocytes of all treated animals received 3 mg/kg sildenafil and more for 28 days and more. Some hepatocytes of sildenafil treated rabbits showed occasional pyknosis specially the necrotic ones and those exhibited hydropic changes (fig. 1).

Necrosis

Occasional necrotic hepatocytes were seen in the liver of rabbits exposed to sildenafil. This alteration was more pronounced within the periportal zone hepatocytes where some of these cells stained more blue than normal (fig. 2).

Hydropic degeneration

Hepatocytes cloudy swelling of sildenafil-treated rabbits was seen. This alteration became more prominent with increasing the dose and duration of sildenafil exposure and related with necrotic hepatocytes (fig. 3).

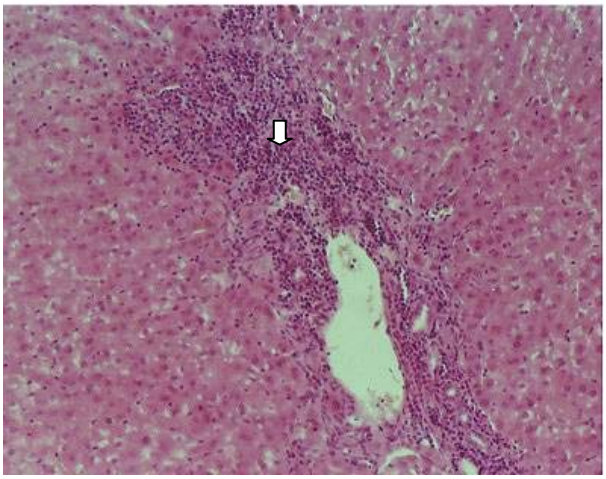

Fig. 5: Sildenafil-treated rabbit received 3mg/kg for 14 days demonstrating inflammatory cell inflammation (arrow). H&E stain.

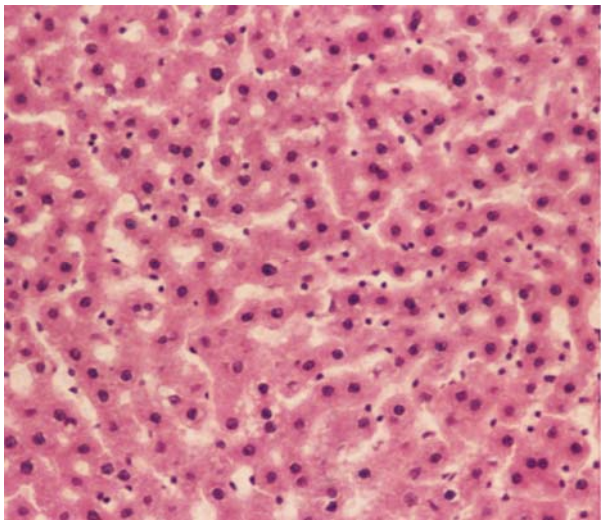

Fig. 6: Sildenafil-treated rabbit received 3mg/kg for 14 days demonstrating Kupffer cell hyperplasia. H&E stain.

Bile duct hyperplasia

Compared with the control animals, dilatation of the bile duct was seen together with proliferation of the lining epithelium in the treated ones (fig. 4). This abnormality was noticed in animals received 6mg/kg sildenafil and more for 28 days and more.

Inflammatory cells infiltration

An aggregation of inflammatory cells filtration mainly in the hepatic portal space was seen with predominance of lymphocytes and plasma cells (fig. 5). This change appeared after 14 days and more of sildenafil exposure.

Kupffer cells hyperplasia

Kupffer cells became more prominent and increased in number in the liver of rabbits exposed to sildenafil. This alteration appeared early at 3 mg/kg sildenafil and more for 14 days and more of drug exposure (fig.6).

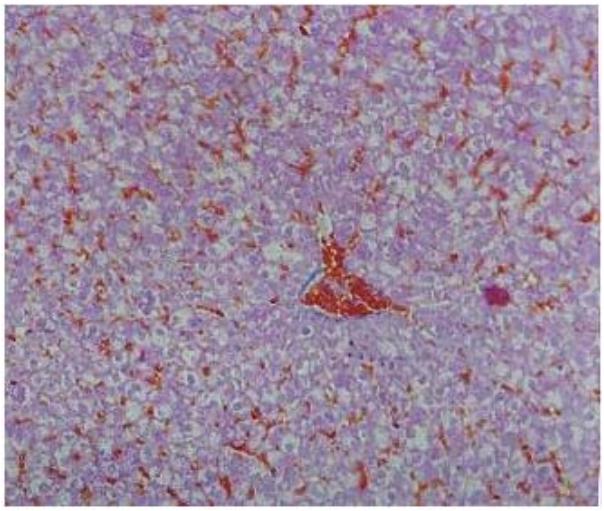

Fig. 7: Sildenafil-treated rabbit received 9mg/kg for 28 days demonstrating congestion of hepatic vessels. Trichrome stain.

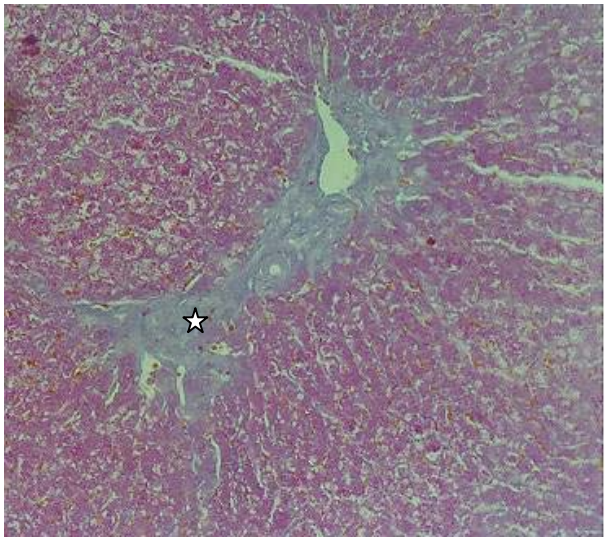

Fig. 8: Sildenafil-treated rabbit received 6mg/kg for 35 days demonstrating portal space fibrosis (star). Trichrome stain.

Congestion of hepatic vessels

Occasional congestion of portal vessel and central veins was observed (fig. 7).

Portal space fibrosis

Slight fibrosis in the portal space was detected (fig. 8). This change was seen mainly in the hepatic tissues of rabbits received 6mg/kg sildenafil and more for 28 days and more.

Glycogen depletion

Compared with the control liver, evident partial heterogeneous glycogen depletion was observed in the hepatocytes of rabbits exposed to sildenafil. This depletion became more evident in the liver of rabbits exposed to 9mg/kg sildenafil for 35 days (figs. 9&10).

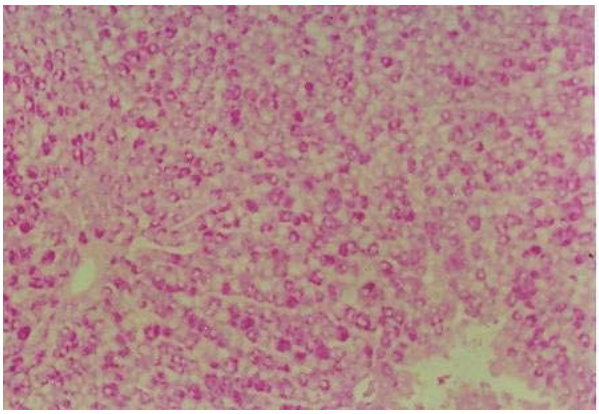

Fig. 9: Control rabbit received a single i.p. injection of sterile 0.45% sodium chloride for 35 days demonstrating normal pattern of glycogen storage. PAS stain.

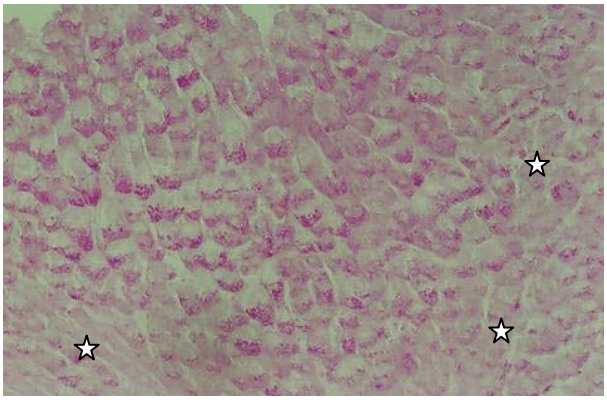

Fig. 10: Sildenafil-treated rabbit received 9mg/kg for 35 days demonstrating glycogen depletion (stars). PAS stain.

No fatty change was detected due to sildenafil in the liver of all rabbits under study. Also, none of the above histological and histochemical changes were seen in the hepatic tissues of the control group. In addition, no mortality was recorded in any of the treated animals of the present work with no change was detected in the behavior and appearance of rabbits exposed to sildenafil.

DISCUSSION

The results of the present work reveal that sildenafil overdoses caused significant alterations in the hepatic tissues. The metabolism of this drug occurs in the liver and excreted by both the liver and kidneys. Some studies indicated that the liver contributes in sildenafil detoxification where the bioavailability of this drug is determined by the pre-systemic first-pass hepatic metabolism (Walker *et al*., 1999; Muirhead *et al*., 2002). Sildenafil is metabolized mainly by the contribution of CYP2C11 to N-desmethyl sildenafil together with glutathione conjugates, that restore reactive oxygen species generation ability leading to drug detoxification by the formation mercapturic acid (Wada *et al*., 2009). The elevation of AST and ALT due to sildenafil overdoses as seen by the present work indicates hepatocytes damage brought about together with amino acid metabolism reduction as well. This biochemical finding is in agreement with the histological ones and with the reduction in the body weight of rabbits received 9mg/kg/body weight of sildenafil which is associated with the time exposure to the drug.

The finding of the present study indicate that sildenafil overdoses intoxication induce hepatocytes nuclear alterations. Anisonucleosis was reported to be associated with hepatic dysplasia and carcinomatous lesion (Zusman *et al*., 1991). This alteration together with binucleation as seen by the present work represents a consequence of cell injury usually seen in regenerating cells and might be related to cellular over activity together with nuclear alterations due to sildenafil detoxification (Gerlyngl *et al*., 2008; Zamzami and Kroemer, 1999). Karyorrhexis and karyolysis were demonstrated by sildenafil overdoses. These alterations are seen usually in cells undergo necrosis or apoptosis and represents nuclear chromatin condensation while karyorrhexis and karyolysis are resulted from destructive fragmentation and chromatin matter dissolution of a necrotic or dying cells (Zamzami and Kroemer, 1999; Kumer *et al*., 2007; Pandey *et al*., 2008). On the other hand, the seen hepatocytes necrosis might indicate glutathione depletion indicated by oxidative stress on the hepatocytes as a result of sildenafil toxicity.

The inflammatory cells infiltration in the liver due to sildenafil exposure may suggest that this drug interacts with the hepatic interstitial tissues. This alteration also indicate an interference of this drug with the mechanism of antioxidant defense which may lead to the generation of reactive oxygen species and enhancement of the seen inflammatory response (Johar *et al*., 2004).

The induced hydropic degeneration by sildenafil overdoses might indicate hepatic tissues injury induced by this drug. This cytoplasmic alteration might be a result of disturbances in ion and fluid homeostasis due to increase in the interstitial and intracellular water (Schrand *et al*., 2010). In addition, this abnormality might indicate that sildenafil overdosing can induce lysosomal hydrolytic enzymes leakage causing cytoplasmic degeneration (Del Monte, 2005). Also this degeneration might be resulted from disturbances in the function of the cellular membranes that cause massive influx of water and sodium ions due to sildenafil overdosing toxicity.

Table 1: Weekly change on the average body weight of rabbits subjected to overdoses of sildenafil for 35 days.

Dose		Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	P-value
Control group (formulated vehicle)	Average body weight (g)	1067±5.1	1089±6.2	1119±6.4	1141±5.2	1155±4.3	1182±6.3	-
	Amount of Change (g)	-	22±0.5	26±0.2	16±0.9	19±0.5	27±1	-
1mg/kg body weight	Average body weight (g)	1083±6.3	1107±5.2	1138±7.2	1162±6.4	1180±8.1	1196±7.7	<0.001*
	Amount of Change (g)	-	24±0.6	31±1	24±0.5	18±0.8	16±0.6	-
3mg/kg body weight	Average body weight (g)	1075±4.4	1084±5.6	1090±4.3	1085±3.9	1081±4.5	1087±5.4	0.695
	Amount of Change (g)	-	9±0.6	6±0.7	-5±0.4	-4±0.3	6±0.5	-
6mg/kg body weight	Average body weight (g)	1094±4.7	1101±4.6	1107±3.4	1102±4.5	1104±5.4	1098±3.2	0.459
	Amount of Change (g)	-	7±0.3	-3±0.6	-5±0.5	2±0.6	-6±0.7	-
9mg/kg body weight	Average body weight (g)	1081±3.6	1090±4.4	1086±4.8	1079±3.9	1074±4.2	1073±4.4	0.232
	Amount of Change (g)	-	9±0.6	-4±0.3	-7±0.7	-5±0.4	-1±0.2	<0.05*

* Statistically significant (p-value<0.05) by using one-way ANOVA test.

Table 2: Amount of change on the relative ratio of liver weight to body weight of rabbits subjected to overdoses of sildenafil for 35 days.

Dose	Average liver weight (g)	Average body weight (g)	The percentage absolute liver weight	Liver index (L_x)
Control group	58.9±2.6	1182±6.3	4.98±0.4	-
1mg/kg body weight	60.1±3.7	1196±7.7	5.2±0.45	1.04±0.09
3mg/kg body weight	53.5±2.1	1087±5.4	4.92±0.43	0.98±0.08
6mg/kg body weight	52.7±2.4	1098±5.2	4.79±0.45	0.96±0.09
9mg/kg body weight	50.8±1.8	1073±4.4	4.73±0.40	0.95±0.07
P-value	-	-	>0.05[a]	>0.05[a]

[a] One-way ANOVA test (p-value <0.05) was used as a statistical analyses tool.

Table 3: Serum biochemical analysis results of rabbits subjected to overdoses of sildenafil for 35 days.

Biochemical test	Control (n=6)	1 mg/ kg/day (n=6)	3 mg/ kg/day (n=6)	6 mg/kg/day (n=6)	9 mg/kg/day (n=6)	P-value
AST (IU/l)	21.52±3.1	27.35±3.4	39.72±4.5	50.63±5.3	55.30±5.6	<0.001*
ALT (IU/l)	9.88±2.1	12.8±2.3	18.03±2.2	23.63±2.1	45.95±2.9	<0.001*
Total Protein (mg/dl)	7.09±0.5	8.56±0.6	8.97±0.6	9.41±0.8	10.4±0.9	<0.001*
Uric acid (mg/dl)	4.4±0.3	4.31±0.4	4.34±0.5	4.41±0.3	4.37±0.4	0.92
Urea (mg/dl)	50.4±4.2	44.47±4.7	33.47±4.1	27.44±3.7	22.81±3.3	<0.001*
Creatinine (mg/dl)	1.56±0.2	1.37±0.2	0.92±0.1	0.84±0.1	0.59±0.1	<0.001*
Testosterone (nmol/l)	6.2±0.9	6.9±0.8	8.2±0.9	8.1±0.9	8.9±0.8	<0.001*
FSH (IU/l)	1.9±0.3	2.3±0.3	4.2±0.3	4.7±0.4	5.7±0.4	<0.001*
Luteinizing hormone (IU/l)	3.2±0.4	3.1±0.5	3.4±0.7	3.3±0.5	3.2±0.3	0.87

* Statistically significant (p-value <0.05) by using one-way ANOVA test.

The results of the present work showed that sildenafil overdoses induced bile duct hyperplasia. This might indicate that this drug and/or its metabolites are excreted via bile secretion with an irritation effect on the bile duct epithelium.

The current findings illustrated that sildenafil overdoses could activate Kupffer cells phagocytic activity as a defense mechanism of detoxification (Neyrinck *et al*., 2004). This might be as a result of increased autophagy throughout the hepatic tissues to remove the accumulated drug and its metabolites and to contribute to the hepatic oxidative stress correlated with the hepatic tissues injury induced by sildenafil overdosing toxicity. On the other hand, the seen congestion in the hepatic blood vessels might be a result of endothelia injury of these vessels due to sildenafil overdosing. Also, The induced depletion of glycogen storage by sildenafil overdoses may indicate

that glucose absorption or the enzymes involved in the process of glycogenesis or/and glycolysis are affected by this drug.

In addition, serum biochemical analysis of the present study showed that sildenafil overdoses elevated significantly testosterone, FSH and total protein. This is in line with the results of other investigations where overdoses of the drug under study had provoked testicular damage and an arrest of spermatogenesis (Jarrar, 2011).

CONCLUSION

The findings of present study indicate that sildenafil overdosing produces considerable hepatic histological alterations and biochemical changes that might affect the function of the liver and other organs. Also, these findings might also indicate a need to study the effect of sildenafil overdosing among patients with hepatic impairment and to investigate sildenafil safety among people including the elderly ones, where liver performance is significantly affected (Koltz, 2009).

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