The Possible Histological Effects of Daclatasvir on Liver Cells of the Pregnant Mice

Original Article

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ABSTRACT

Introduction: HCV infection is a worldwide health problem. Egypt is one of the highest prevalence in adults. The prevalence of infection in pregnancy is between 1% and 8%. Treatment during pregnancy has been associated with many problems. Unfortunately, till now there is no approval for an antiviral drug to be used in pregnancy. A new group of antiviral agents has emerged known as directing acting antivirals (DAAs). Daclatasvir (DCV) is one of the second generation DAAs that has pangenotypic activity.

Aim of the Work: Was to assess the possible histological effects of daclatasvir on the liver cells of the pregnant mice.

Materials and Methods: Forty adult pregnant female mice were divided into two main groups; Group I (control group), included 10 mice which received distilled water. Group II (DCV group): included 30 mice subdivided into three equal groups: group IIa (DCV 12.5 mg/kg), group IIb (DCV 25 mg/kg) and group IIc (DCV 50 mg/kg body weight). They received the drug orally from GD6 to GD15. The pregnant mice were sacrificed (at GD 18-19) under anesthesia and after taking blood samples for biochemical analysis. Liver specimens were processed for histological and morphometric analysis.

Results: Maternal toxicity with increased maternal mortalities was observed. Common hepatocellular changes in the three experimental groups showing; disturbed hepatic architecture, hypereosinophilic foci with indistinct boundaries, nuclear alterations, cellular vacuolation and prominent Von-kupffer cells. The presence of basophilic foci was prominent in GIIc. Increased collagen deposition and high levels of liver enzymes were mostly prominent in GIIa more than GIIc and GIIb respectively. These findings were concomitant with idiosyncratic drug induced liver injury (DILI) with pregnancy.

Conclusion: DCV administration during pregnancy has a dose-dependent potential risk of DILI. Future studies are recommended to improve maternal outcome.

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Key Words: Daclatasvir; HCV; hepatotoxicity; liver; pregnancy.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a worldwide health problem that affects nearly 2%-3% population in the world[1,2]. The Egyptian Health Issues Survey (EHIS) was conducted in 2015 to re-estimate Egypt's prevalence of HCV infection after it was initially estimated in 2008. It was observed that The HCV antibody prevalence was 10.0% for the ages 15–59 and the HCV RNA prevalence was 7.0%.The prevalence of HCV antibody and HCV RNA was 0.4% and 0.2% respectively in children aged 1–14 years. About 3.7 million people in 2015 have chronic hepatitis C viral infection in the 15–59 age groups. The prevalence of HCV RNA has been estimated to decrease by about 29% since 2008[3].

Women account for about 35.8% of cases of chronic HCV cases[4]. The course of HCV and its complications differ from female to male. However, women with HCV face special risks related to antiviral therapy during pregnancy and lactation and the potential to transmit their offspring vertically[5].

The prevalence of infection in pregnancy is between 1% and 8%. For antibody positive women, the vertical transmission rate is 1.7% and for women with HCV viremia documented, the total rate estimated to be 4.3%. Females co-infected with HIV/HCV have a rate of 19.4% regarding vertical transmission[6].

Pregnancy is characterized by physiological adjustments in the maternal compartment. Some investigators clarified the influence of pregnancy on maternal liver in CD-1 mice. They revealed that maternal liver enlargement during pregnancy is a growth response induced by hepatocyte proliferation and also identified concomitant changes in hepatic gene expression[7,8].

Dai G *et al*.,[8] reported an increase in the numbers of mitotic figures of hepatocytes, an indicator of hepatocytes undergoing cell division, indicating cellular hyperplasia during pregnancy.

They reported also hepatocyte hypertrophy as a contributor to maternal liver mass expansion during

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pregnancy. Taken together, they concluded that pregnancy-induced hepatomegaly is a physiological event of liver growth underlying which are hepatocyte hyperplasia and hypertrophy[8].

Additionally, liver regeneration is accompanied by hepatic apoptosis[9]. To determine whether this event also occurs during maternal liver growth, hepatic caspase 3 protein was examined in non-pregnant and pregnant mice. Unexpectedly, although total caspase 3 protein levels showed an increase during the first half of pregnancy, cleaved caspase 3 was not detected at all the time points examined. The data suggest that apoptosis may not be a major event during pregnancy-induced maternal liver growth[8].

Pregnancy induces widespread maternal changes in the structures and functions of virtually every organ system. These physiological changes represent essential maternal adaptations to meet the needs of the development and growth of the placenta and fetus[10].

Pregnancy-induced changes in liver function tests have been reported in humans, including alterations of serum albumin and bilirubin levels, aspartate transaminase and alanine transaminase activities, and triglyceride and cholesterol concentrations in the blood[11].

HCV antiviral therapy aims at achieving a sustained virological response or permanent viral clearance as assessed by negative HCV viral load 6 months or more after the treatment course has been completed. HCV therapy is a rapidly evolving field, with many new drugs coming onto the market. Regimen selection now depends primarily on viral genotype, cirrhosis status, and history of previous exposure to treatment[12].

In 2011, the Food and Drug Administration approved the first generation of direct-acting antiviral drugs (DAAs), boceprevir and telaprevir. These drugs improved interferon and ribavirin efficacy but with significantly greater inconvenience, pill burden, adverse effects, and financial costs[12].

The second generation of direct-acting antiviral drugs (DAAs), including sofosbuvir, simeprevir, and ledipasvir, has greatly improved treatment outcomes and side-effect profiles compared to earlier drugs. Currently approved regimens for HCV management are the same for both men and women. Outcomes of treatment are similar between both sexes[5].

Treatment of the infected pregnant females has associated problems that are not rare. Thus, pregnancy is the 3rd most common contraindication for treatment, and postponed treatment onset in approximately 2% of HCV infected patients in the developed countries, as the Unites States of America[13].

Pregnant women face special treatment concerns regarding to the teratogenic potential of HCV antivirals. Some anti-HCV drugs (like ribavirin and interferon α) are contraindicated during pregnancy because they interferes with the development of the embryo and are embryotoxic or embryolethal in animal models. Drug manufacturers recommend avoiding use of these drugs in pregnancy and during the next 6 months following exposure for both females and their male partners[5].

Till now, there is no approval for an antiviral drug to be used in pregnancy and/or lactation. Thus, little is reported about the effects of anti-HCV drugs during pregnancy and/or lactation[14].

A future goal regimen is to be well tolerated, interferon free, ribavirin (RBV) free, and having a pangenotypic action is always needed to be developed in the future. Such a regimen may be a combination of different DAAs with various mechanisms of actions[15].

The targets of DAAs are the non-structural (NS) proteins of the HCV particles that are required for viral replication which are NS3, NS5A and NS5B. Combinations of two or more DAAs reduce the risk for emergence of resistant strains of the virus[15].

In 2015, the second generation DAAs was available. The pharmacokinetic properties of this generation are not clearly understood, and further data are needed for full comprehension of this generation properties. Owing to the lack of data on the impact of this generation on pregnancy, physicians could only expect that the physiological changes that take place during pregnancy may influence the peak plasma concentration and drug metabolism. These drugs were reported to have the ability to easily cross the placenta[16].

The directly acting antiviral daclatasvir (DCV) is one of the second generation drugs of DAAs. It is a NS5A inhibitor, which is active against different HCV genotypes[17]. NS5A inhibitors inhibit the viral RNA replication and inhibit viral release[18].

Daclatasvir is considered to be the primary NS5A complex inhibitor that is approved to be used as a part of combination regimens with Sofosbuvir, Ribavirin, and PEG-IFN for treating chronic viral hepatitis C in adults regarding the European Union[16].

The drug bioavailability after oral administration is high, with plasma concentrations matching dose to 60 milligrams and steady state 3-4 days following dosage. Daclatasvir is highly protein bound (99%) regardless of the dose, with 10 times lower tissue concentration[19].

DCV is a substrate for P-glycoprotein and its metabolism is mainly by the cytochrome P450 (CYP450) 3A4. These metabolites represent about 5% of the unchanged drug found in the plasma[20].

Fatigue, headache, pruritus, rash and nausea are the commonly observed adverse drug reaction for all arms. Elevated alanine aminotransferase (ALT), total bilirubin, and anemia are the laboratory abnormalities of interest, and these are comparable for all the treatment arms[20].

There is scanty in the available data on the use of the new generation of anti-HCV oral drugs (DAAs) in pregnant mice. Moreover, DCV has been recently approved by FDA for human use in 2016. The available studies on the drug are only concerned with its use in management of HCV infection and its associated complications. Therefore, there is no available database assessing its use in pregnant liver tissue.

The aim of the present study was to estimate the possible histological effects of daclatasvir on the liver cells of the pregnant mice.

MATERIALS AND MATERIALS

The present study was conducted at both Embryology lab in Anatomy and Embryology department and the Center of Excellence for Research in Regenerative Medicine Applications (CERRMA) at Alexandria Faculty of Medicine (AFM). The experimental procedures followed the code of research ethics approved by the Research Ethics Committee.

Material and equipment used

Test drug

Daclatasvir dihydrochloride (Daktavira 60 mg ®) tablets, a product of European Egyptian Pharmaceutical Industries, Alexandria, Egypt. Daclatasvir dihydrochloride is easily soluble in water. The control group received distilled water obtained from El-Gomhorreya Company, Alexandria, Egypt.

Chemicals for skeletal and histological examination

1. H&E stain (Biodiagnostic Company, Cairo, Egypt).
2. Trichrome stain (Biodiagnostic Company, Cairo, Egypt).
3. Osmic stain (Biodiagnostic Company, Cairo, Egypt)

Animals

Forty pregnant mice (CD-1) strain, approximately eight weeks old (adult) weighting (25-30 g), were obtained from the Animal House center, Physiology Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt.

Equipment

Light microscope (CERRMA lab, Faculty of Medicine, Alexandria University) was used to examine deparaffinized sections of the liver specimens of the pregnant mice.

Methods

I. Animals husbandry and matting procedure

Mice were examined for the health status and acclimatized to the laboratory environment for two weeks prior to the study. The animals were housed, in cages about four/cage, with controlled light at photoperiods of 12 hours light and 12 hours dark and a temperature around 27 ± 5°C. They were given standard diet and water throughout the study period.

To set up the mattings, females in each group were examined in the afternoon and those in estrus phase (swollen corrugated vaginal opening) are placed into the cages with males (two females/ one male per cage). The female mice were examined for the presence of vaginal plug on the next morning. Once the vaginal plug has appeared, this day was considered to be Gestational day 0 (GD 0).The pregnant mice were isolated in separate cages (4/cage) and followed up for pregnancy signs[21].

II. Study groups and Experimental procedure

The forty pregnant mice were divided into two main groups:

- Group I (control group): included 10 mice that received 1 ml distilled water.
- Group II (DCV group): included 30 mice which were subdivided into three equal groups according to the dose of daclatasvir, 10 mice each, that received oral daily daclatasvir dihydrochloride as follow:
    1. Group IIa: 12.5 mg/kg body weight.
    2. Group IIb: 25 mg/kg body weight.
    3. Group IIc: 50 mg/kg body weight.

From GD6 (6 days after the appearance of the vaginal plug) to GD15 the pregnant mice in each group received oral daily treatment by oro-gastric tube. The drug used in the tablet form. Tablets were crushed to make a powder form then dissolved in distilled water and received in the form of an oral suspension of the drug calculated according to the body weight of mice.

The least dose (12.5mg in group IIa) was adjusted to be equal to the human equivalent daily dose of 60.9 mg /day. The human recommended daily dose of daclatasvir was reported to be 30-90mg /day[22].

The animal doses of drug were calculated by estimating the human equivalent dose (HED) according to the equation reported earlier: animal dose (mg/Kg) = human equivalent dose (mg/Kg) × 12.3[23,24], considering that an average human adult weight is 60 Kg.

Liver dissection and processing for histological examination[25].

The pregnant mice were sacrificed (at GD 18-19) after inhalation of Diethyl ether. The livers of sacrificed animals were dissected. The right lobes were taken and fixed in 10% buffered formalin, embedded in paraffin and sectioned. Sections were sliced at a thickness of 5µm. Staining included hematoxylin-eosin (H&E) and Gomori's trichrome to assess fibrosis

For assessment of lipid content in the hepatocytes, the left lobes were divided into thin slices of tissue and fixed in Flemming's fluid. Sections were rinsed in running

water for 12-16 minutes then dehydrated in 70%, 90% and absolute alcohol. Sections were cleared with xylene then choloroform and embedded in paraffin wax. Sections were cut at 5-10µ, mounted on slides and left to dry. Wax was removed by xylene and mounted with synthetic resin medium.

Morphometric study

Morphometric studies were done by NIH Image J software[26]. Images from Gomori's trichrome stained sections were viewed and recorded using Olympus microscope – equipped with Spot digital camera with numerical aperture of a high resolution (16-bit digital camera (1280X1024 pixels). The photographs of liver tissue of each group of mice were captured at magnification 10X object lens. Five randomly selected fields of each group were measured using computer program MAtLAB software (image J, the MATHWORKS, inc., USA). Measurements were expressed in the form of percentage area using NIH Image J software[26]. Such data were subjected to biostatistical analysis.

Biochemical study of Liver Function Tests[27]

Before scarification of pregnant mice, blood samples were taken from the inferior vena cava (IVC) in plain tube vacutainers then left for 30 min to clot. Serum was separated within one hour after centrifugation at 2000g for 10 min. Sera from all groups were kept at -800C then an automated chemical analyzer was used regarding the manufacturers' instructions to estimate SGOT and SGPT level.

Statistical analysis

The biochemical and morphometric results were analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). The Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data was expressed as mean ±standard deviation (SD). Significant differences were determined by using ANOVA and pairwise comparison (between each 2 groups) was done using Post Hoc Test (Tukey). Values were considered significant when $P<0.05$[28].

RESULTS

Maternal mortality: seven deaths of the pregnant mice were encountered in study groups; two mice in group IIa at GD 12, 13; two mice in group IIb at GD7,8; and three mice in group IIc at GD 7,8.

Histological results

a) H& E Stain

Examination of the control mice liver (G I) revealed the classical structure with cords of hepatocytes of one or two cell thickness radiating from central veins and separated by blood sinusoids (Figure 1-a).

The hepatocytes were polyhedral with granular hypereosinophilic cytoplasm and vesicular nuclei. Some bi-nucleated cells were also noticed.

The blood sinusoids were lined by flattened endothelial cells and kupffer cells. Portal tracts were encountered at the margin of the classical lobules with branches of portal vein, hepatic artery and bile duct (Figure 2-a).

Examination of the liver samples of group IIa mice (received 12.5mg DCV) revealed definite degenerative changes with obvious centri-lobular pattern and disturbed hepatic architecture. Dilated central veins with cellular debris were depicted surrounded by cellular masses separated by irregular distribution of widened sinusoids (Figure 1-b).

The hypereosinophilic foci showed multiple cells with indistinct boundaries, glassy homogenous cytoplasm, and dark small nuclei. Some cells showed vacuolations.

Alternating areas with basophilic small cells with irregular small dark nuclei were also depicted. The dilated sinusoids were lined by flattened endothelial cells and prominent Von kupffer cells loaded with brownish pigmentation. Many cells with flattened nuclei were seen bordering some of these sinusoids (Figure 2-b).

Examination of the liver samples of group IIb mice (received 25 mg DCV) revealed partially preserved hepatic architecture with slightly vacuolated eosinophilic cells with vesicular nuclei arranged in cords radiating from central veins and separated by sinusoidal spaces. Foci of vacuolated crowded clear cells with disturbed sinusoidal arrangement were frequently encountered (Figure 1-c).

These vacuolated cells are larger in size as compared to neighboring cells with just a hint of pale eosinophilic staining, highly vacuolated cytoplasm and eccentrically or marginated small dark nuclei. Some of these cells were ruptured with indistinct boundaries. Others appeared with signet shape and peripherally pushed nuclei.

The nuclei depicted variable affection. Some nuclei were pale with multiple nucleoli, or small size darkly stained, others showed chromatolysis. The occasionally encountered dilated sinusoids revealed prominent kupffer cells (Figure 2-c).

Examination of the liver samples of group IIc mice (received 50 mg DCV) revealed widely disrupted hepatic architecture with prominence of clear cellular foci with obliterated unnoticed sinusoids (Figure 1-d).

Overcrowded clear vacuolated cells with remnants of eosinophilic cytoplasmic stain were seen surrounding dilated central veins loaded by cellular debris, eosinophilic contents and blood cells. The vacuolated cells were large, some show indistinct boundaries with ill-defined small pleomorphic dark nuclei mostly marginated.

Few eosinophilic cords of hepatocytes separated by sinusoids were occasionally encountered. Other foci of small dark basophilic cells with pleomorphic dark nuclei were rarely encountered. Prominent Von kupffer cells were depicted in the occasionally observed sinusoidal spaces (Figure 2-d).

b) Gomori's Trichrome stain

Control mice liver (group I) revealed the classical distribution of collagen fibers within the hepatic architecture. Few stained fibers were seen within the portal tract and around the central veins (Figure 3-a).

Excessive fibrosis was seen in livers of group IIa mice with densely stained fibers in areas of portal tracts and around, central veins and in between hepatocytes (Figure 3-b). However, intercellular fiber deposition was noticed on examination of group IIb (Figure 3-c) and group IIc (Figure 3-d) as well as around the central veins.

c) Osmic stain

Stained black fat droplets were seen scattered in the cytoplasm of control livers' hepatocytes. The cells showed moderate amount with intercellular variation in fat content (Figure 4-a).

Group IIa livers revealed less loaded hepatocytes with fat content showing excessive clear vacuolation (Figure 4-b). Group IIb hepatocytes revealed wide areas of poor stained disrupted cells alternating with few remnant loaded cells (Figure 4-c). Group IIc hepatocytes revealed overcrowded cells with moderately stained cytoplasm. Some cells showed variable degrees of vacuolation (Figure 4-d).

Morphometric analysis

Morphometric analysis for collagen fibers deposition in Gomori's trichrome stained liver sections was done for the study groups. Digital images were obtained from Gomori's trichrome stained sections using a digital camera connected to the microscope (Olympus B x41) at magnification x 100 (n=5/group). Measurements were expressed in the form of percentage area using NIH Image J software.

The mean area percentage of collagen fibers showed a significant difference in between groups of the study. It was significantly increased in DCV groups as compared to the control one (p <0.001).

By comparing different groups of study, the mean area percentage of collagen fibers was; 43.51, 37.10 & 44.87 in DCV received groups GIIa, GIIb, & GIIc respectively compared to 22.42 in the control group (Table 1).

There was a significant increase in collagen deposition in the three experimental groups compared to the control (p <0.001). A non-significant increase in deposition of collagen between GIIa with GIIb & GIIc (p1=0.057, p2=0.932). However, a significant increase of collagen deposition was depicted by comparing GIIb & GIIc (p3=0.018)

Biochemical analysis of liver Enzymes

In the current study, the mean levels of serum SGPT and SGOT showed a significant increase in the experimental groups compared to the control (p<0.001for both SGPT & SGOT).

By comparing experimental groups regarding SGPT values, a non-significant difference between GIIa and GIIb (p1=0.999). There was a statistically significant difference between GIIc with GIIa and GIIb (p2 & p3<0.001).

Regarding SGOT values, there was also non-significant difference between GIIa and GIIb (p1=1.000). However, a statistically significant difference between GIIc with GIIa and GIIb (p2=0.038, p3=0.038) (Table 2).

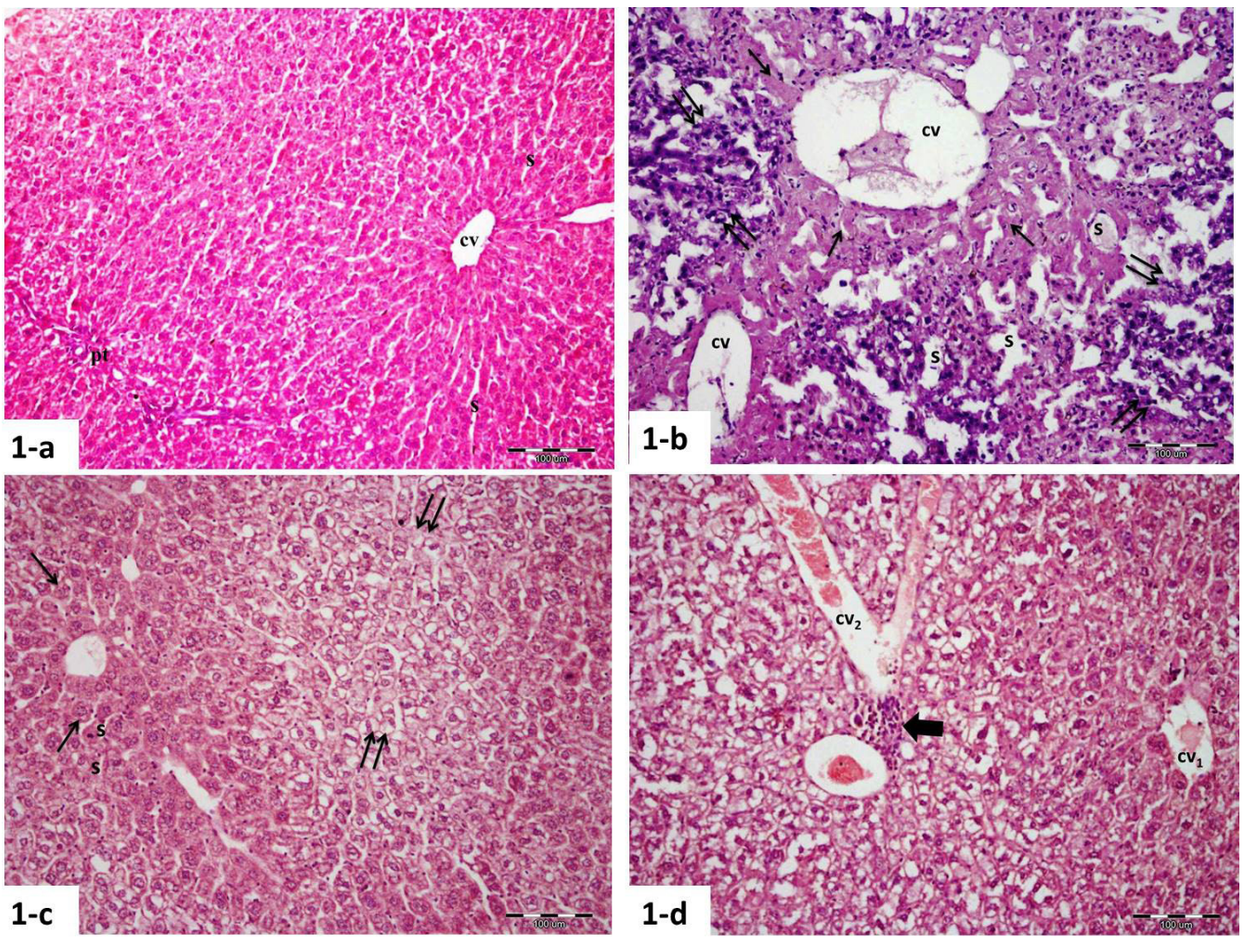

Fig. (1a-d): Photomicrographs of livers of mice in study groups showing:
a) The classical structure of the control livers with cords of hepatocytes radiating from central veins (CV) separated by blood sinusoids (s). A portal tract (pt) with branch of portal vein and bile duct is also seen.
b) Group IIa mice livers (received 12.5 mg DCV; showing multiple degenerative changes with centrilobular affection and destruction of normal architecture. Dilated central veins (cv) with cellular debris are frequently seen. Hyper-eosinophlic foci of multiple cells with glassy homogenous cytoplasm (↑) and loss of boundary are seen bordering irregularly arranged widened sinusoids (s). Other areas (↑↑) show basophilic foci of cells with dark irregular nuclei.
c) Group IIb mice livers (received 25 mg DCV) showing; preserved hepatic architecture with highly vacuolated eosinophilic cells depicting vesicular nuclei (↑) bordering the hepatic sinusoids (s). Foci of highly vacuolated clear cells with just a hint of pale eosinophilic staining highly vacuolated cytoplasm and eccentrically located or marginated small dark nuclei (↑↑) are depicted.
d) Group IIc mice livers (received 50 mg DCV) showing disturbed architecture with obliterated sinusoids. Overcrowded cells surrounding dilated central veins with cellular debris (cv1) or congested (cv2). A localized focus of small sized basophilic cells with small dark nuclei (thick arrow) is also seen.
H&E stain Mic Mag bar 100 µm.

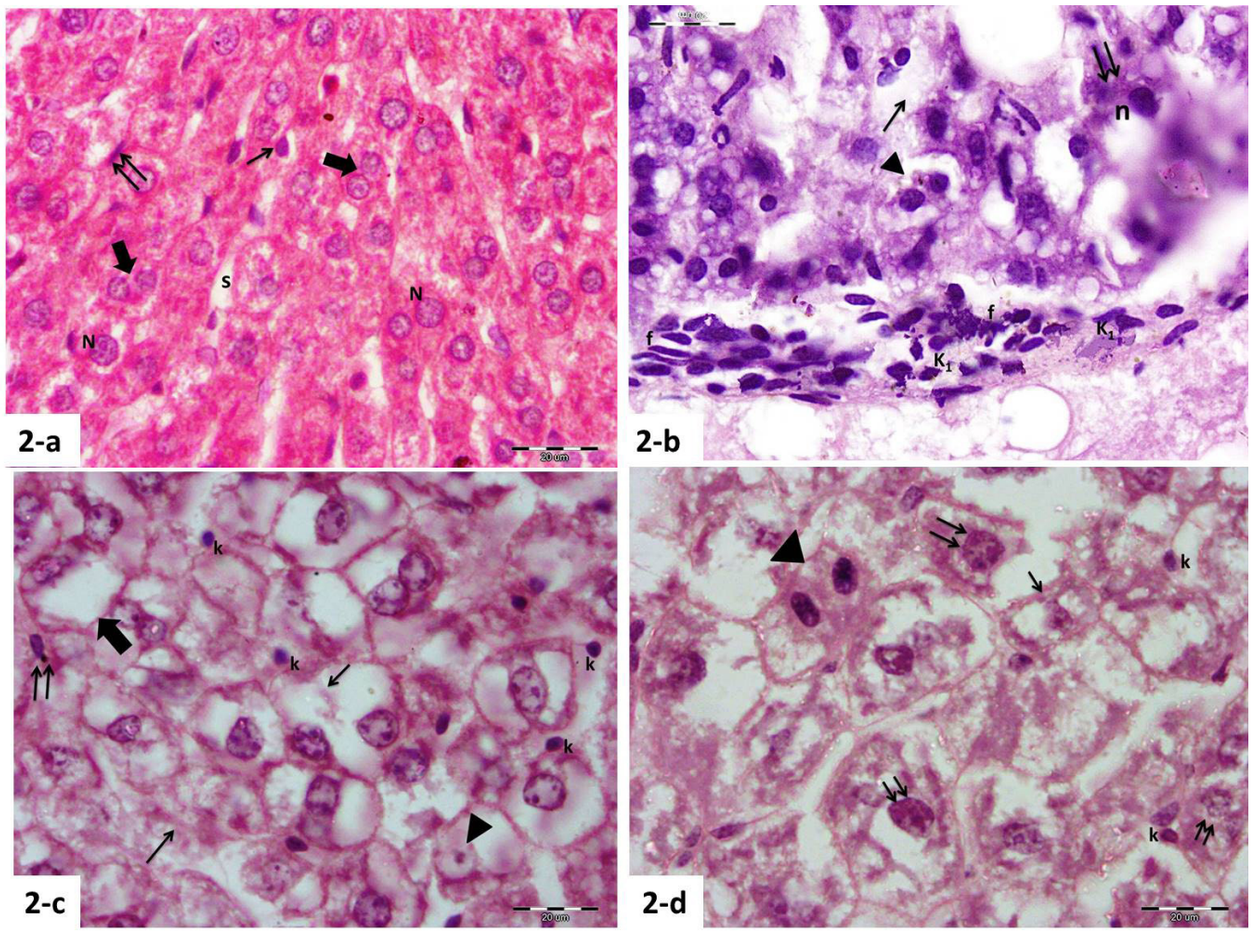

Fig. (2a-d): High power magnification of livers of mice in study groups showing;
a) Hepatocytes of the control mice showing; polyhedral hypereosinophilic cells with vesicular nuclei (N) arranged in cords of one or two cell thickness and bordering sinusoids (s) lined by endothelial cells (↑↑) and kupffer cells (↑). Some binucleated hepatocytes are also seen (thick arrow).
b) Group IIa livers showing; disturbed architecture with indistinct hepatic cords. Alternating foci of eosinophilic (↑) and basophilic (↑↑) cells with vacuolated cytoplasm (▲) and dark nuclei (n) are seen. Irregularly arranged widened sinusoids are occasionally seen with prominent Von kupffer cells overloaded by brown pigments (k1). Many cells with flattened nuclei (f) are seen bordering some of the sinusoids.
c) Group IIb liver showing; overcrowded ballooned hepatocytes with unnoticed sinusoids. Some of these cells are ruptured with indistinct boundaries (↑). Others show chromatolysis (▲) or dark shrunken nuclei (↑↑). Few cells showed signet shape with marginated nucleus (dark arrow). The cytoplasm is highly vacuolated in most of the cells and the nuclei show multiple nucleoli. Many kupffer cells (k) are seen within the remaining sinusoids.
d) Group IIc showing; overcrowded cells with no apparent sinusoids. The hepatocytes are of different shape and size. Some show indistinct boundaries with ill-defined small nuclei (↑). Others show nuclear pleomorphism with multiple nucleoli (↑↑). Binucleated cells with dark smaller nuclei (▲) are also seen. Von kupffer (k) are depicted within the seen sinusoids.

H&E stain Mic Mag bar 20 µm.

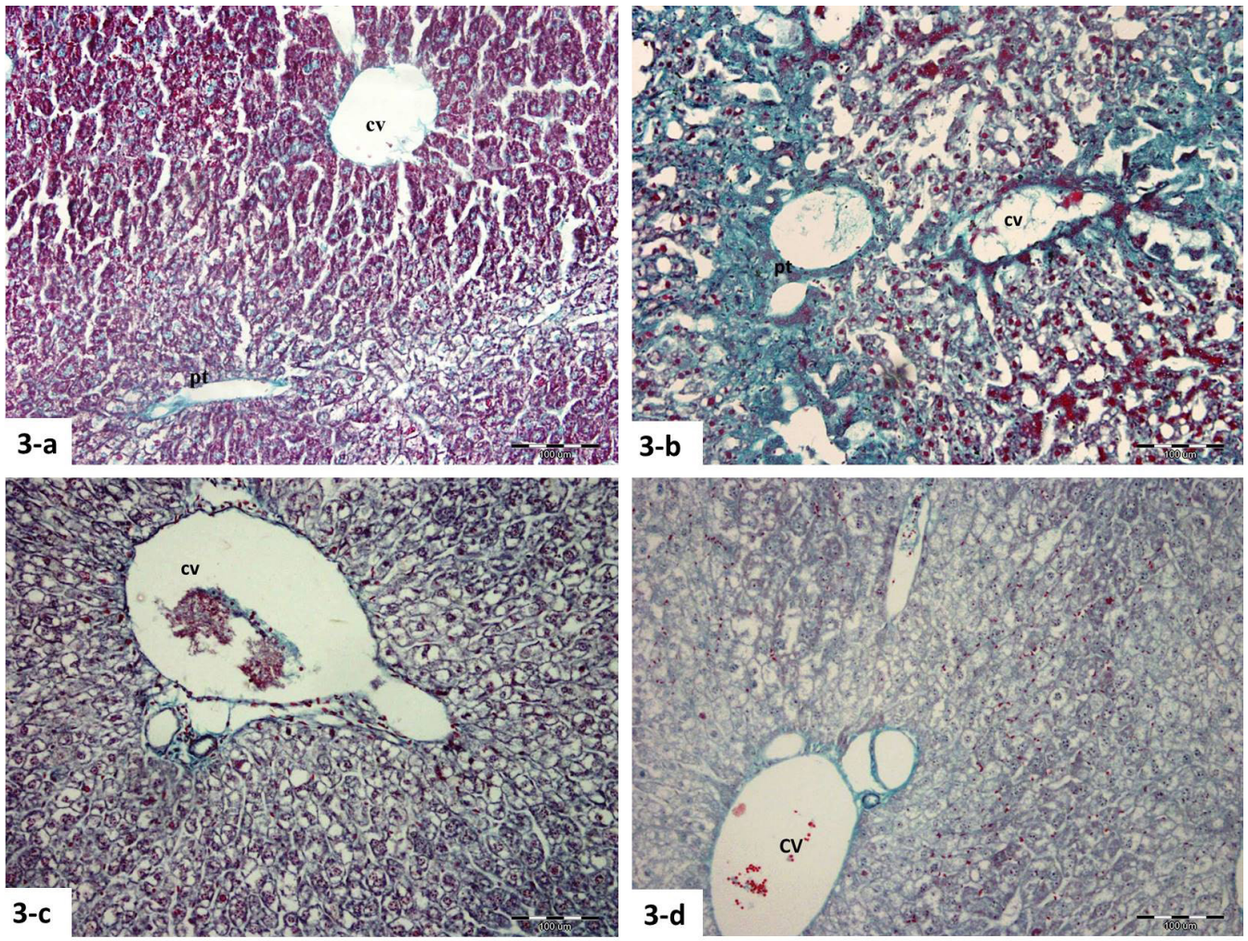

Fig. (3a-d): Photomicrographs of livers' samples stained by trichrome stain showing green stained collagen fibers in the portal tract (pt) and around central veins (cv). Note the excessive staining in (b) especially around dilated central veins. Moderate reaction is seen in (c&d).
a) Control group I
b) Group IIa
c) Group IIb
d) Group IIc

Trichrome stain Mic. Mag x bar 20 µm

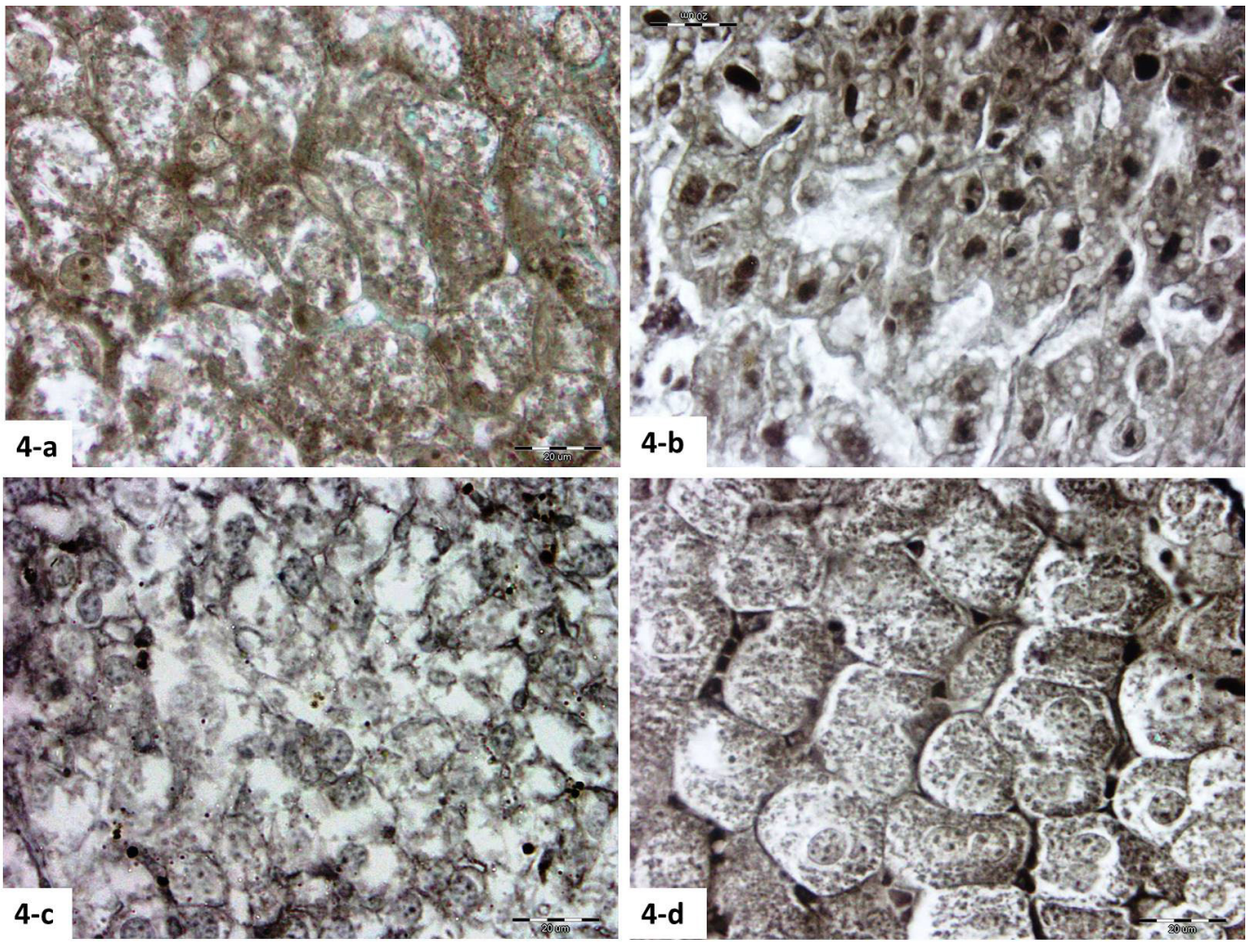

Fig. (4a-d): Photomicrographs of livers' samples stained by osmic stain showing: black stained fat droplets within the hepatocytes:
(a) The control mice cells show variable lipid content.
(b) GIIa hepatocytes show less loaded fat content with vacuolated cells with disturbed arrangement
(c) GIIb cells show areas of unstained disrupted vacuolated cells.
(d) GIIc cells show variable lipid content with some loaded cells and other pale stained cells.

Osmic stain Mic. Mag x bar 20 µm.

Table I: Comparison between the different studied groups according to % area of collagen fibers deposition in Gomori's trichrome stained liver sections

Percentage of Area	Group I (Control) (n = 5)	Group II			*P*
		IIa (DCV 12.5) (n = 5)	IIb (DCV 25) (n =5)	IIc (DCV 50) (n = 5)	
Min. – Max.	18.45 – 27.64	36.04 – 49.0	36.1 – 38.9	42.3 – 48.2	
Mean ± SD.	22.42 ± 4.63	43.51 ± 4.91	37.10 ± 1.1	44.87 ± 2.5	<0.001*
Median (IQR)	20 (19.1 – 27.3)	45 (41.7 – 45.8)	36.9 (36.8–36.8)	45 (42.9 – 46.6)	
p0		<0.001*	<0.001*	<0.001*	
Sig. bet. grps.		p1=0.057, p2=0.932, p3=0.018*			

IQR: Inter quartile range
p: *p value* for comparing between the studied groups
p0: *p value* for comparing between control and each other groups
p1: *p value* for comparing between IIa and IIb
p2: *p value* for comparing between IIa and IIc
p3: *p value* for comparing between IIb and IIc
*: Statistically significant at $p \leq 0.05$

Table II: Comparison between the four groups of the study according to liver enzymes (SGPT & SGOT)

Biochemical analysis	Control (n = 10)	Experimental			*P*
		IIa (DCV 12.5) (n = 10)	IIb (DCV 25) (n =10)	IIc (DCV 50) (n = 10)	
SGPT (u/ml)					
Min. – Max.	13.0 – 18.0	25.0 – 49.0	24.0 – 48.0	14.0 – 17.0	
Mean ± SD.	15.30 ± 1.42	40.20 ± 9.81	39.80 ± 10.34	15.60 ± 1.17	<0.001*
Median (IQR)	15.0(15.0 – 16.0)	46.0(30.0 – 48.0)	45.0(26.0 – 47.0)	15.50(15.0 – 17.0)	
p0		<0.001*	<0.001*	1.000	
Sig. bet. grps.		p1=0.999, p2<0.001*, p3<0.001*			
SGOT (u/ml)					
Min. – Max.	40.0 – 45.0	62.0 – 69.0	63.0 – 69.0	32.0 – 66.0	
Mean ± SD.	42.0 ± 1.76	66.60 ± 2.32	66.60 ± 1.96	59.30 ± 11.08	<0.001*
Median (IQR)	42.0(40.0 – 43.0)	67.50(65.0 – 68.0)	67.0(65.0 – 68.0)	64.0(62.0 – 65.0)	
p0		<0.001*	<0.001*	<0.001*	
Sig. bet. grps.		p1=1.000, p2=0.038*, p3=0.038*			

IQR: Inter quartile range
p: *p value* for comparing between the studied groups
p0: *p value* for comparing between control and each other groups
p1: *p value* for comparing between IIa and IIb
p2: *p value* for comparing between IIa and IIc
p3: *p value* for comparing between IIb and IIc
*: Statistically significant at $p \leq 0.05$

DISCUSSION

DAAs are recently considered to be the drug of choice in all of management protocols of HCV patients with chronic hepatitis and liver cirrhosis. The target of treatment is to induce complete viral clearance followed by relieve of hepatocellular inflammation, regression of fibrosis, liver cirrhosis and its subsequent complications.

The present study is targeting the drug induced hepatocellular effects of DCV; one of the most commonly used DAAs in treatment protocols of patients suffering from chronic HCV infection. The unique feature of the current study is the presence of pregnancy as a confounding variable in studying DCV effect on normal liver of the pregnant mice.

The study groups depicted gross changes in the livers of the dissected mice alternating between; localized areas of pallor in the liver GIIa and areas of minor hemorrhages in the liver of GIIc.

Histological findings: The three groups of the study that had received DCV revealed a group of changes in the form of; disturbed hepatic architecture, hypereosinophilic foci with indistinct boundaries, cellular vacuolations, prominent Von kupffer cells loaded with brownish pigmentation. These findings were described to be reversible cellular changes according to Robbins and Cotran, Pathologic Basis of Disease[29].

Cellular boundaries were indistinct and some of these cells showed ruptured cell membrane that was explained to be an early sign of hepatocyte necrosis and irreversible cell death[29].

The nuclei revealed variable affection. Some nuclei were pale with multiple nucleoli, or small size darkly stained or even showed chromatolysis. Eccentrically or marginated pleomorphic rounded nuclei, and signet shaped nuclei were also depicted. All of these could be explained to be signs of hepatocellular damage induced by the drug administration[29].

Livers of group IIb mice revealed a different finding in the form of foci of vacuolated crowded clear cells with disturbed sinusoidal arrangement. Group IIc mice showed foci of small dark basophilic cells with pleomorphic dark nuclei.

These focuses of cellular change have been prescribed as small to large hepatocyte aggregates within the hepatic parenchyma which could be putative preneoplastic lesions[30].

Unfortunately, the animal studies assessing DAAs histological effects are scanty. Most of the available literatures on these drugs are clinical studies or case reports on DAAs as a treatment protocol in managing chronic viral hepatitis and its complications.

On the other hand, the majority of available literatures on DAAs have reported suppression of inflammation of liver tissue on patients suffering from chronic HBV and HCV infection. They currently announced that, interferon or DAA-based antiviral treatment protocols have improved hepatocellular inflammation, and this was proved by biomarkers[31-33] and paired liver biopsies[34-36].

They also reported decreased level of ALT after treatment with DAAs that indicates suppression of liver inflammation and hepatocellular damage[37]. It was

supposed that; the improvement of liver inflammation, as indicated by biomarkers and biopsies taken from the liver, was the result of complete viral clearance rather than the effect of the treatment itself[34,38,39].

Recently, the U.S. Food and Drug Administration has warned about the potential for serious liver injury in patients with advanced liver conditions as a result of the direct-acting antivirals use for management of hepatitis C Virus[40].

A case report study that agreed with the results of the present study; has demonstrated a link between the concomitant use of DAAs and hepatotoxicity[41].

Two cases of major hepatotoxicity related to sofosbuvir and NS5A therapy have been reported. In Case 1; reported an acute hepatocellular damage after two weeks of receiving sofosbuvir, ledipasvir and ribavirin[41].

In case 2; a marked worsening of synthetic liver function was reported after around three weeks after administration of sofosbuvir, daclatasvir and ribavirin. In this case report, the authors couldn't exclude the possibility of hepatocellular damage due to unknown interaction or drug reaction[41].

Hasin *et al*.[40] also reported another case report of drug induced liver injury associated with administration of DAAs. The case has received ombitasvir, paritaprevir, ritonavir, Dasabuvir and ribavirin (PODr + R). Deterioration of liver functions has been noted after two weeks of drug administration. On day 24, she stopped anti-hepatitis C treatment and the case has got gradual recovery. The diagnosis was suspected to be DILI.

A recently published case control study conducted in Egypt to study the effect of DAAs on decompensated cases of liver cirrhosis. Hanafy *et al*.[42] reported a link between DAAs therapy for 3 months and the risk of HCC appearance. They reported an increased risk of HCC by 10% in cases within 6 months of treatment with DAAs[42]. This is in agreement with the findings of the current study regarding the appearance of suspicious preneoplastic lesions in group IIb and IIc after administration of one of DAAs continuously for 10 days in pregnant mice.

The current work has reported an excessive collagen deposition indicating liver fibrosis shown by Gomori's Trichrome staining. The examined liver specimens of group II c& a mice have depicted an excessive collagen deposition in areas of portal tracts, around central veins and in between hepatocytes. However, more intercellular fiber deposition was noticed by examination of group IIb and group IIc as well as around the central veins.

This has been confirmed by morphometric analysis for collagen fibers deposition in Gomori's trichrome stained liver sections. The mean area percentage of collagen fibers showed a significant difference in between groups of the study. The mean area of collagen deposition was highest in groups IIc and IIa respectively.

Unfortunately, there is no available data on non-pregnant healthy livers to compare the results of the present study regarding liver fibrosis. The available data is only concerned with the use of DAAs in management of HCV associated liver fibrosis and cirrhosis.

On contrary to the current work, a group of studies have assessed liver fibrosis following DAAs therapy using noninvasive measures. They reported amelioration in biomarker-based liver fibrosis[43]. Other studies have also declared liver stiffness improvement after DAA therapy. This was assessed by following the patients at the end of treatment course, six months, and one year following termination treatment course[43-45].

The authors have explained the improvement of liver fibrosis initially by amelioration of inflammation followed by decrease fibrosis progression[46].

Up till now, data on liver fibrosis changes after DAA therapy are still very limited, with a relatively short follow-up. Therefore, only interferon-based studies can specify the real effects. As interferon evidence shows, regression or resolution of fibrosis occurs but is a slow process that takes years[39,47].

Biochemical analysis of liver enzymes in the current study showed a significant increase in SGPT (ALT) and SGOT (AST) levels in the experimental groups compared to the control. The mean of SGPT measurements were lower than those of SGOT, with the maximum in GIIa followed by GIIb and GIIc.

This was concomitant with the morphometric analysis of collagen deposition in these groups respectively. This can be explained according to Robbins and Cotran[29] by hepatocellular damage following DCV administration for ten successive days.

However, the current work revealed that the least dose of DCV (GIIa) has showed the highest increase of liver enzymes and excessive collagen deposition as indicated by Gomori's trichrome stain, morphometric and biochemical analysis.

Collectively, the results of the current work indicating that; GIIa showed signs of hepatocellular damage by the drug (H&E stain) in addition to periportal fibrosis (Gomori's Trichrome) and clear cellular vacoulation (osmic stain)[29]. GIIb showed also hepatocellular damage with early stage of cirrhosis as indicated by disturbed sinusoidal arrangement (H&E), intercellular fibrosis (Gomori's Trichrome) and wide areas of cellular vacoulation alternation with areas loaded with fat (osmic stain). GIIc depected hepatocellular damage, cirrhotic changes indicated by disturbed sinusoidal arrangement (H&E) and intercellular collagen deposision and around central vein (Gomori's Trichrome)[29] In addition, GIIb and GIIc depicted foci of cells with the appearance of suspicious preneoplastic lesions after DCV administration[30].

The low dose resulted in liver damage in the way of liver fibrosis while the increased dose stimulated the cirrhosis

pathway which was dose dependent and G III manifested greater damage and more obvious precancerous alteration. Thus it is dose dependent.

Let us put another theory that the results of this study can be can explained by induction of Drug-induced liver injury (DILI) associated with pregnancy. This explanation is based on the fact that, there is no other factor associated with pregnancy in the current work except for DCV administration. This type of DILI can be explained to be an idiosyncratic effect to the drug.

This idiosyncratic type of DILI affects rare sensitive persons with lower doses and variable characteristics. Its diagnosis is mainly dependent on the basis of the exclusion of other causes as no specific markers have been identified[48].

Based on the previous explanation, DCV can affect the liver in a different way regardless the dose of the drug. Therefore, the severity of liver affection by the drug is not related to the dose of the drug.

Although pregnancy is considered to be one of the main host factors predisposing to idiosyncratic DILI, scanty of available literatures discussing DILI associated with pregnancy till now[49]. Unfortunately, DILI was reported to be one of the major causes of acute hepatic failure in the developed countries. It is responsible for around 50% of cases of acute hepatic failure. Also, pregnant mothers with elevated LFT were found to have DILI in 2%-3% of cases[50].

Lao T.[49] has reported an association between poor perinatal outcomes and DILI in pregnancy. Subsequently, good understanding of DILI was recommended in order to improve future maternal and perinatal effects[49]. An alarming point regarding DILI in pregnancy is that, there is no association between teratogenicity and DILI. Some safely administered drugs have showed idiosyncratic DILI[51].

Cano Paniagua A and Amariles P[52] have reported the adverse effect of antimicrobial agents on liver during pregnancy. A significant hepatotoxicity and even acute fulminant hepatitis were associated with the use of ART during pregnancy[53].

Snijdewind *et al*.[54] agreed with the results of the present study regarding the association between elevated liver enzymes and antiviral intake during pregnancy. They reported a higher incidence of elevated level of liver enzymes in pregnant compared to non-pregnant females. There was also a higher incidence of mild compared to severe degree of liver enzyme level elevation[54].

Finally, the reported maternal mortality in the current study may be caused by an acute hepatocellular damage associated with DCV administration as a result of sever form of DILI. This explanation needs more confirmation in the future studies on the same subject.

CONCLUSION

DCV administration during pregnancy is associated with a potential risk of DILI. This type of DILI could be explained to be an idiosyncratic effect of the drug in pregnant mice. This may be the result of immune-modulatory effect of pregnancy on hepatic tissue.

Subsequent studies in the same field are highly recommended due to the scanty of available information, in addition to the positive impact on improving maternal outcome. This can be achieved by a better awareness of how to medically manage the pregnant women.

ABBREVIATIONS

HCV: Hepatitis C virus, **EHIS:** Egyptian Health Issues Survey, **RNA:** Ribonucleic acid, **HIV:** Human immunodeficiency virus, **DAAs:** Direct-acting antiviral drugs, **RBV:** Ribavirin, **NS:** Non-structural, **NS3:** Non-structural protein 3, **NS5A:** Non-structural protein 5A, **NS5B:** Non-structural protein 5B, **DCV:** Daclatasvir, **PEG-IFN:** Pegylated interferon, **CYP450:** Cytochrome P450, **ALT:** Alanine aminotransferase, **CERRMA:** Center of Excellence for Research in Regenerative Medicine and its Applications, **AFM:** Alexandria Faculty of Medicine, **H&E:** Hematoxylin and Eosin stain, **GD:** Gestational day, **HED:** Human equivalent dose, **NIH:** National Institute of Health, **Image J:** Java-based image, **MAtLAB:** Matrix laboratory, **inc:** Incorporated company, **IVC:** Inferior vena cava, **SGOT:** Serum glutamic oxaloacetic transaminase, **SGPT:** Serum glutamic pyruvic transaminase, **SPSS:** Statistical Package for the Social Sciences, **SD:** Standard deviation, **ANOVA:** Analysis of variance, ***P value:*** Probability value, **CV:** Central veins, **s:** Sinusoids, **pt:** Portal tract, **N:** Nuclei, **K:** Kupffer cells, **Mic Mag:** Microscopic magnification, **IQR:** Inter quartile range, **Min:** Mminimum, **Max:** Maximum, **HBV:** Hepatitis B virus, **PODr + R:** Ombitasvir, paritaprevir, ritonavir, Dasabuvir and Ribavirin, **DILI:** Drug induced liver injury, **HCC:** Hepatocellular carcinoma, **AST:** Aspartate aminotransferase, **LFT:** Liver function test, **ART:** Antiretroviral drugs

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التأثيرات الهيستولوجيه المحتمله للدكلاتاسفير على خلايا الكبد في حوامل الفئران

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المقدمة: تعتبر عدوى الإلتهاب الكبدي الفيروسي مشكلة صحية عالمية، وتعد مصر من أعلى معدلات الإنتشار بين البالغين. و يتراوح معدل إنتشار العدوى أثناء الحمل بين ١٪- ٨.٪. و لإرتباط العلاج أثناء الحمل بالعديد من المشكلات، فإن تناول الدواء أثناء فترة الحمل غير مصرح به حتي الآن. و قد ظهرت مؤخرا مجموعة جديدة من الأدويه المعالجه للفيروسات الكبديه تعرف بإسم (الأدوية المضادة للفيروسات الموجهة) و يعتبر دواء الدكلاتاسفير أحد أفراد الجيل الثاني لهذه المجموعه، والذي يمتلك تأثيراً واسعاً يشمل جميع انواع الفيروسات الكبديه.

الهدف: تقييم التأثيرات الهيستولوجية الكبديه المحتملة الناتجه عن إعطاء الداكلاتاسفير لحوامل الفئران.

مواد و طرق البحث: تم تقسيم عدد ٤٠ من حوامل الفئران إلى مجموعتين رئيسيتين: المجموعة الأولى (المجموعة الضابطة)، تضمنت ١٠ من حوامل الفئران والتي تلقت الماء المقطر. وتضمنت المجموعة الثانية ٣٠ من حوامل الفئران تم تقسيمها إلى ثلاثة مجموعات متساوية: تلقت الأولي (أ) ١٢,٥ مجم/كجم ، والثانيه (ب) ٢٥ مجم/كجم، والثالثه (ج) ٥٠ مجم/كجم من وزن الفأر من عقار الدكلاتاسفير. تم إعطاء الدواء عن طريق الفم من اليوم ٦-١٥ من الحمل. تم ذبح الفئران الحوامل بعد التخديرفي اليوم ١٨-١٩ من الحمل بعد أخذ عينات الدم للتحليل الكيميائي الحيوي. ثم معالجة عينات الكبد للتحليل النسيجي والقياسات الشكليه الكميه لها.

النتائج: أظهرت النتائج تأثير سمي مع إرتفاع معدل الوفيات لحوامل الفئران. كما أظهرت مجموعه من التغيرات الكبدية في المجموعات البحثيه الثلاث (أ ، ب، ج) كما يلي؛ إضطراب المعالم الكبدية، وجود تجمعات خلويه غير واضحة الحدود، و تغيرات متعدده في أشكال الأنويه و تركيبها بالخلايا ، مع وجود فراغات خلويه، و زياده عدد خلايا الخلايا الاكوله بالكبد (فون كوبفر). كما لوحظ وجود تجمعات من الخلايا ذات الصبغه القاعدية في كبد الفئران بالمجموعه (ج). مع زيادة ترسب الكولاجين خاصه فى المجموعه البحثيه أ و التى اظهرت ايضا مستويات عالية من إنزيمات الكبد مقارنة بكل من مجموعه (ج) و (ب) على التوالي. وقد كانت هذه النتائج متوافقة مع التأثير السمي للدواء علي خلايا الكبد أثناء فترة الحمل.

الإستنتاج : إستخدام الدكلاتاسفير أثناء فترة الحمل يؤثر تأثيراً سلبيا على خلايا الكبد ويرتبط التأثير السمى بالجرعه المستخدمه. ولذلك يوصى بدراسات مستقبلية في نفس المجال لتحسين النتائج النهائيه لعلاج الأمهات أثناء فترة الحمل.