



Possible Role of Angiogenesis Suppression in Rats Model of Non-alcoholic Fatty Liver Disease

Dalia M. A.G. Rezk, Mohammed E.M. Sarhan, Hanaa A. Abdel Moneim , Amr M. Abbas and Mohammed E.D.

Salama

Medical Physiology Department, Mansoura faculty of Medicine, Mansoura, Egypt

Received: 17 July. 2018

Accepted: 15 Feb 2019

Available online: 27 April 2019

Keywords

- Angiogenesis
- Non alcoholic fatty liver disease (NAFLD)
- Sorafenib
- Vascular endothelial growth factor (VEGF)

Abstract

Objective: to evaluate development of angiogenesis in rat model of NAFLD and to determine the possible protective effects of antiangiogenic therapy (sorafenib) in preventing the progression of NAFLD. **Methods:** A total of 45 Male Albino rats (200-300 g) were divided into 3 groups (15 rats for each): **Group I:** Control group fed on an ordinary diet. **Group II:** rats received high fat, high fructose diet (HFD,HFr) with DEN (10mg/kg body weight twice weekly for 8 week, ip). **Group III:** rats received HFD, HFr + DEN + sorafenib (2.5 mg/kg/d orally for 8 weeks). Biochemical, histopathological, and immunohistopathological examination were studied. **Results:** Administration of high fat, high fructose diet (HFD,HFr) with DEN resulted in a significant elevation in the serum levels of cholesterol, TG, LDL, AST, and ALT, significantly lower serum levels of HDL and Albumin together with a significant decrease in hepatic GSH when compared with normal control rats. Histopathological examination with (H & E) revealed that liver of untreated rats showed severe fatty infiltration (grade3). Immunohistochemical examination of liver of untreated NAFLD rats showed strong staining reactions against VEGF, α -SMA, CD31, and Caspase3 monoclonal antibodies. Oral administration of sorafenib alleviated all these distorted parameters. **Conclusion:** Angiogenesis revealed another pathological mechanism accelerating the NAFLD progression to liver fibrosis. Also, antiangiogenic agent (Sorafenib) suggested as effective preventing therapy that limits NAFLDs progression by inhibiting angiogenic process.

Corresponding author: Dalia M. A.G. Rezk, Medical Physiology Department, Mansoura faculty of Medicine, Mansoura, Egypt,

Telephone: 0502546592, **Email:** dodh_doc@yahoo.com

INTRODUCTION

Non alcoholic fatty liver disease (NAFLD) simply includes non alcoholic fatty liver (NAFL) has simple steatosis without inflammation and its progressive form, non alcoholic steatohepatitis (NASH), with inflammation and hepatocellular injury with or without fibrosis (1).

In recent years, it has been found that the prevalence of NAFLD reached approximately 20-30% in general population in the western world, it reaches approximately up to 70% of type 2 diabetes mellitus (DM). However, in Asia the prevalence of NAFLD was ranged from 15% to 30% in the general population and over 50% in patients with DM and metabolic syndrome. 20-30% of NASH patients developed cirrhosis, and 30%-40% of patients with NASH cirrhosis had risk for liver-related death (2).

Pathogenesis of progression of NAFLD is multifactorial, two-hits hypothesis is well recognized, **the first hit** involves imbalance of fatty acid metabolism that leads to hepatic fatty infiltration (steatosis). **The second hit** may be oxidative or metabolic stress as mitochondrial dysfunction, endoplasmic reticulum stress, and increased proinflammatory cytokines leading to subsequent inflammation (steatohepatitis) and fibrosis. Liver fibrosis occurs as a compensatory response to the process of tissue repair in NAFLD. During the progression of NASH, activated **hepatic stellate cells (HSCs)** are profibrogenic hepatic cells by deposition of extracellular matrix (ECM) (3).

During chronic inflammation, hypoxic liver tissue by fatty deposition and capillarization or dedifferentiation of liver sinusoidal endothelial cells (LSECs). Hypoxic liver tissue causes up-regulation of VEGF, Angiotensin and their receptors in hepatic stellate cells, enhancing the hypoxia-induced angiogenesis. Receptors for VEGF (VEGFRs) are expressed in liver sinusoidal endothelial and stellate cells. Also, capillarized LSECs synthesize platelet-derived growth factor (PDGF) and transforming growth factor β (TGF β), therefore they may contribute to angiogenesis with chronic inflammatory hepatic state (4).

Sahin et al. found that VEGF, the master regulator of angiogenesis, is also implicated in fibrogenesis by activation of HSCs (5). LSECs also release angiocrine signals balancing liver regeneration and fibrosis. With acute injury, chemokines such as stromal derived factor (SDF)-1 are upregulated to initiate regeneration, this response will shift to maladaptive (pro-fibrotic) angiocrine response with chronic injury by persistent activation of fibroblast growth factor receptor1 (FGFR1) (6).

Studying the possible association of angiogenesis in progression of NAFLD may provide a new perspective to reveal another pathological mechanism (7). **Sorafenib** is multikinase inhibitor mediates antiangiogenesis by inhibiting both VEGF and PDGF receptors. Also, it reduces the number of activated HSC, inflammation, and angiogenesis (8).

Although, it is still unclear whether angiogenesis represents a simple response to

maintain homeostasis and repair hepatic injury (9) and angiogenesis suppression aggravates hepatic fibrosis (10), or angiogenesis exerts a pathological role leading to liver injury, and interference with angiogenesis might be a potential target to avoid fibrosis progression (7). **Therefore, our work aimed to** assess presence of angiogenesis in NAFLD and to evaluate the possible role of antiangiogenic therapy in preventing the progression of non alcoholic fatty liver disease in Albino rats.

Materials and Methods

Chemicals: Diethylnitrosamine (DEN) and Thiopental was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sorafenib (Sorafenib) was purchased from Cipla (Mfd. By CIPLA LTD. Verna Indl. Estate, INDIA)

Animals: This study was conducted on 45 adult male Albino rats weighing 200-300 grams. Animals were bred and housed in the animal laboratory of physiology department, Faculty of medicine, Mansoura University. All experimental protocols were approved by our local ethics committee in May, 2015. Animals were fed a standard laboratory chow with other advised diet with free access to tap water.

Study groups:

Rats were assigned to three groups (15 rats for each group):

Group (i) (Negative control): 15 rats fed on an ordinary diet (60 % carbohydrates, 3 % proteins, 0.8% fats with water, fibers, and minerals) (11).

Group (ii) (NAFLD): 15 rats fed on a HFD, HFr (52% carbohydrate, 24% fat, and 6% protein in form of "15.5% ordinary chow diet, 20% beef tallow, 17.5 % fructose, 39.5% milk powder, and 2.5% NaCl with water, fibers, and minerals" (11)

+ DEN (10mg/kg body weight (ip) twice weekly for 8 weeks) (12). **Group(iii) (Sorafenib group):** 15 rats fed on a high fat, high fructose+ DEN + sorafenib (2.5 mg/d/kg body weight by oral gavage for 8 weeks) (8).

Measurements of biochemical parameters:

Lipid profile (total cholesterol, HDL, LDL and triglycerides) were determined by Enzymatic Colorimetric Kits, **Serum ALT and AST** levels were determined using ALT and AST Activity Assay Kits all of these kits were purchased from Sigma Chemical Co. (St. Louis, MO, USA). **Assessment of Albumin** was achieved using specific kits purchased from Stanbio Lab. (Texas, USA). **Reduced Glutathione (GSH)** assessment was achieved by colorimetric method using specific kits purchased from "Biodiagnostic Co., Egypt".

Histopathological examination

Liver specimens were obtained for histopathological examination. These specimens were placed in plastic cassettes and sunken in neutral buffered formalin for one day. Five-micron thick paraffin sections were prepared, stained with Hematoxylin and Eosin (13). Grades of steatosis according to % involvement of parenchymal fatty infiltration: <5% steatosis was represented as "grade 0", 5-33% steatosis was represented as "grade 1", >33-66% steatosis was represented as "grade2", and >66% steatosis was represented as "grade3" (14).

Immunohistochemical detection:

For CD31 using specific kits of Monoclonal Mouse Antibody CD31, Endothelial cell, Clone JC70A from Dake, Glostrup, Denmark. **Alpha-**

Smooth Muscle Actin (α -SMA): using primary Monoclonal Mouse Antibody against N- terminal synthetic decapeptide of α -Smooth Muscle Actin code M0851, clone 1A4, from Dako, USA.

Vascular endothelial growth factor (VEGF): using VEGF Monoclonal Mouse Antibody against recombinant VEGF protein, clone VG1, code JH121, from ThermoFisher scientific, USA.

Caspase3: using specific kits of Monoclonal Mouse Antibody, code CPP32, Clone 3CSP03, from ThermoFisher scientific, USA.

Computer Assisted digital image analysis (Digital morphometric study): Slides were photographed using Olympus[®] digital camera installed on Olympus[®] microscope with 1/2 X photo adaptor, using 20 X objective. The result images were analyzed on Intel[®] Core I3[®] based computer using VideoTest[®] Morphology[®] software (Russia) with a specific built-in routine stain quantification. Five slides from each case were prepared, 5 random fields from each slide were analyzed. Apply area measurement routine to obtain results expressed as % area of positively stained area in relation to all field area.

Statistical analysis

The obtained data were represented as Mean \pm SD. Comparison for parametric data was done by analysis of variance (ANOVA) followed by turkey's post hoc analysis. $P < 0.05$ was considered significant.

Results

Untreated NAFLD group had significantly higher serum levels of Cholesterol, TG, and LDL with significantly lower serum levels of HDL when compared with normal control group. Treatment with Sorafenib caused a significantly

decrease in serum levels of Cholesterol, TG, LDL, significantly higher levels of HDL when compared with untreated NAFLD rats (Table 1).

Liver enzymes (AST, ALT) were significantly high level and Albumin was a significant decrease in untreated NAFLD rats when compared with control group. Sorafenib administration significantly reduced serum levels of liver enzymes (AST, ALT), and significantly higher levels of Albumin when compared with untreated NAFLD rats (Table2).

There was a significant decrease in hepatic GSH in Untreated NAFLD rats when compared with normal control rats. With Sorafenib administration, there was a significant increase in hepatic GSH when compared with untreated NAFLD group (Table2).

Histological Examination:

Figure1 (a,b, and c) showed hepatic morphology by H&E examination in all groups. Liver of normal control group showed normal hepatocytes with normal radial arrangement around central vein (H&E) (figure1a). Liver of untreated NAFLD group showed severe fatty infiltration (more than 66% parenchymal involvement, grade 3) with coalesced fat droplets, steatosis is predominantly macrovesicular with liver cell injury and focal areas of necrosis (figure1b). However, Sorafenib treated group showed marked improvement with mild hepatic fatty infiltration described as mild steatosis (5-33% parenchymal involvement, grade1) with normal hepatocytes (figure1c).

Table 1: Lipid profile (Cholesterol, triglyceride (TG), HDL, and LDL) in control group (GI), untreated NAFLD group (GII) and sorafenib group (GIII):

Groups(n=15 for each)	Cholesterol (mg/dl)	TG (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Control (GI)	145.06 ± 4.1	115.06 ± 3.78	48.66 ± 0.81	74.52 ± 2.08
NAFLD(GII)	304.66 ± 9.24 **	277.73 ± 4.39 **	25.2 ± 0.84 **	220.76 ± 2.5 **
Sorafenib(GIII)	204.6 ± 2.64 ## **	173.46 ± 2.56 ## **	36.6 ± 0.45 ## *	131.2 ± 1.46 ## **

Result was expressed in the form of mean ± standard error, One way ANOVA with Turkey post hoc test, (HDL) high density lipoprotein, (LDL) low density lipoprotein.

* Significant (P≤0.05); ** Significant (P≤ 0.001).; P: as compared with control group

Significant (P1≤ 0.001), P1: as compared with NAFLD group.

Table 2: Liver enzymes (AST and ALT), Albumin and hepatic GSH among all groups; Control group (GI), Untreated NAFLD group (GII) and Sorafenib group (GIII)

Groups (n=15 for each)	AST (U/L)	ALT (U/L)	Serum Albumin (g/dl)	GSH (mmol/g.tissue)
Control (GI)	25.33 ± 1.25	27.8 ± 1.28	4.59 ± 0.15	7332.33 ± 31.27
NAFLD (GII)	84.73 ± 1.29 **	85.8 ± 1.81 **	2.366 ± 0.157 **	3364.73 ± 33.32 **
Sorafenib(GIII)	63.4 ± 1.67 # *	69.26 ± 1.35 # *	3.1 ± 0.07 # **	4408.8 ± 46.88 ## **

Result was expressed as mean ± standard error. One way ANOVA with Turkey post hoc test, (ALT) Alanine transaminase, (AST) Aspartate transaminase, (GSH) reduced glutathione.

* Significant (P≤0.05); ** Significant (P≤ 0.001).; P: as compared with control group. # Significant (P1≤0.05); ## Significant (P1≤ 0.001), P1: as compared with NAFLD group.

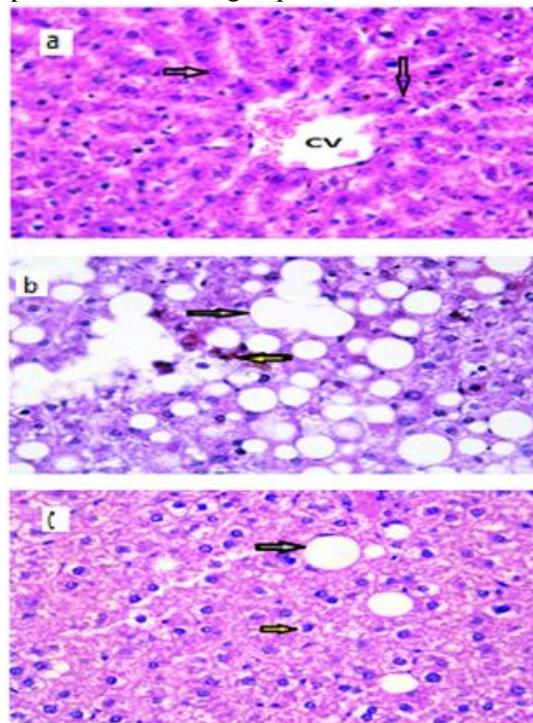


Fig (1 a, b, and c) : Histopathological examination of hepatic parenchyma (1-a) Liver of normal control (GI) showed normal hepatocytes with normal radial arrangement (arrow, ⇨) around central vein(CV). **(1-b)** Liver of untreated NAFLD rat (GII) showed severe fatty infiltration (arrow, ⇨) with areas of focal necrosis (⇩), grade3 steatosis. **(1-c)** Liver of sorafenib treated group (GIII) showed marked improvement with slight fatty infiltration (arrow, ⇨) with normal hepatocytes(⇨), grade1 steatosis (H&E x400).

Immunohistochemical examination of liver against monoclonal antibodies of VEGF, CD31, α -SMA, and Caspase 3 in all groups:

Liver of normal control group showed no staining reaction against VEGF, α SMA, and Caspase3 monoclonal antibody, and weak staining reaction against CD31 monoclonal antibody as shown in **Figures 2a, 3a, 4a, and 5a** respectively. Also, liver of untreated NAFLD showed strong staining reaction against VEGF, α -SMA, CD31, and Caspase 3 as shown in **figures 2b, 3b, 4b, and 5b** respectively with significantly increase in % affected area quantified by image analysis when

compared with normal group as shown in **Table3**.

Figures 2c, 3c, 4c, and 5c showed that liver of Sorafenib treated group showed no staining against VEGF, and Caspase3 monoclonal antibody, weak staining reaction against CD31 monoclonal antibody, and reaction against α -SMA monoclonal antibody respectively but immune reaction against α -SMA restricted to wall of vessels in portal tract, not within liver parenchyma. These findings were confirmed by significantly decrease in % affected area quantified by image analysis when compared with untreated NAFLD group as shown in **Table 3**.

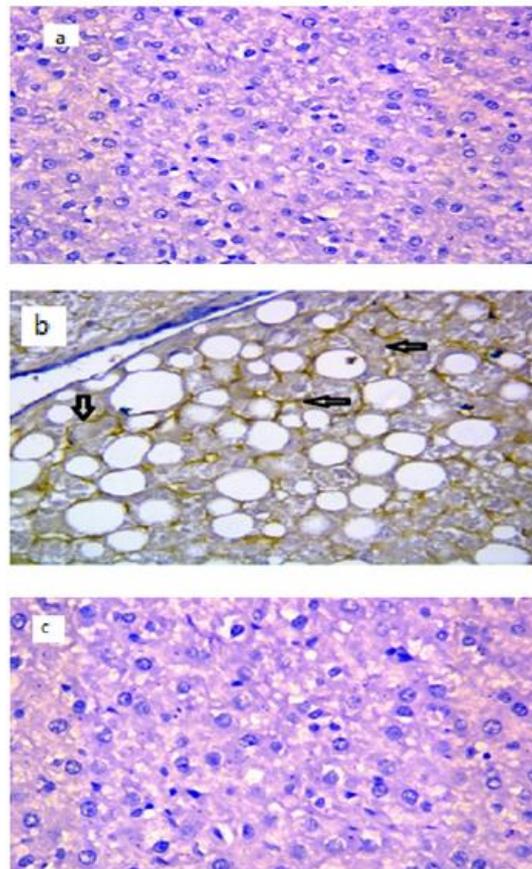


Fig (2a, b, and c): Immunohistopathological examination of liver specimens against VEGF monoclonal antibodies, Liver specimens showed that (2a) Liver of control group (GI) shows no staining reaction against VEGF monoclonal antibody. (2b) NAFLD group (GII) showed strong staining reaction (brown staining) (arrows , \Rightarrow) against VEGF antibody. (2c) Sorafenib group (GIII) showed no staining reaction against VEGF monoclonal antibody.

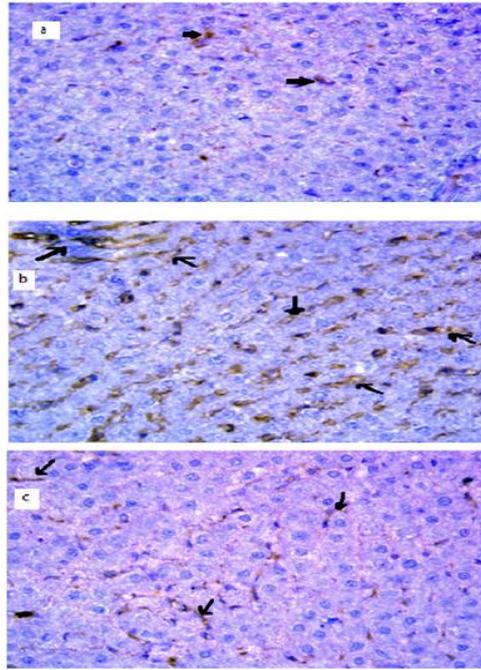


Fig (3 a, b and c): Immunohistopathological examination of liver specimens against CD31 monoclonal antibodies showed that: **(3a)** Control group (GI) showed very weak staining reaction (arrows, \blacktriangleright) against CD31 monoclonal antibody. **(3b)**NAFLD group (GII) showed strong staining reaction, brown staining (arrows, \blacktriangleleft), against CD31 monoclonal antibody. **(3c)** sorafenib group (GIII) showed weak staining reaction against CD31 antibody (CD31 immune staining, x400).

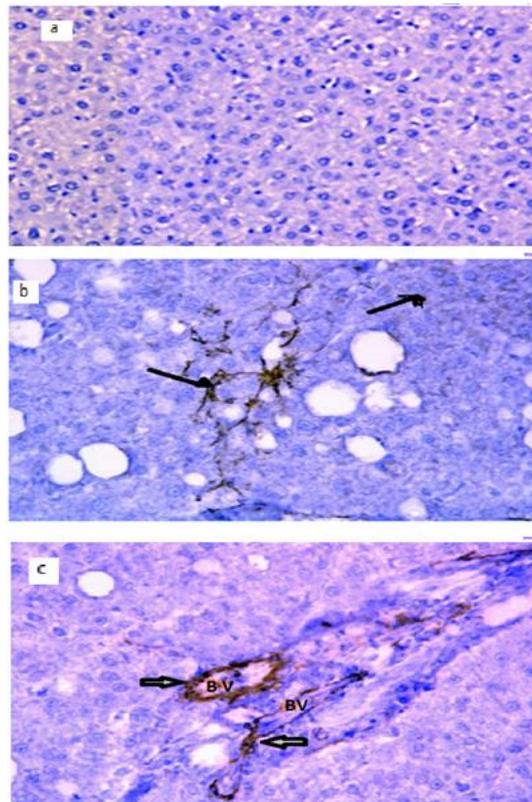


Fig (4 a, b and c): Immunohistopathological examination of liver specimens against α SMA monoclonal antibodies showed that **(4a)** Liver of control group (GI) showed no staining reaction against α SMA monoclonal antibody. **(4b)** Liver of NAFLD group (GII) showed strong staining reaction (arrows, \blacktriangleleft), against α SMA monoclonal antibody, abnormally within hepatic parenchyma near the sites of fatty infiltration. **(4c)** Liver of sorafenib group (GIII) showed appearance of staining reaction (arrows, \blacktriangleright) against α SMA monoclonal antibody restricted to site of blood vessels (BV) (α SMA immune staining, x400).

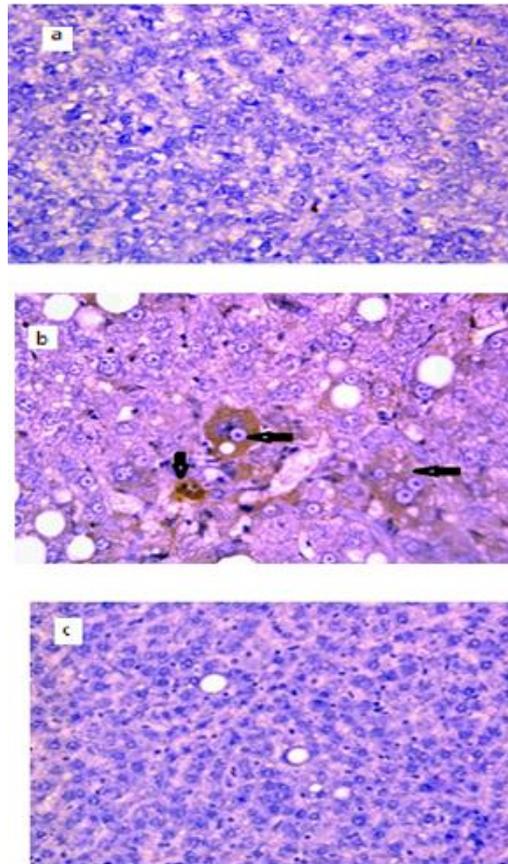


Fig (5 a, b and c): Immunohistopathological examination of liver specimens against caspase3 monoclonal antibodies showed that: **(5a)** Control group (GI) showed no staining reaction against caspase3 monoclonal antibody **(5b)** Liver of NAFLD group (GII) showed brown color indicates immune-positivity. There is strong staining reaction (arrows, \blackrightarrow) against caspase3 monoclonal antibody especially near the sites of fatty infiltration. **(5c)** Liver of sorafenib group (GIII) showed no staining reaction against caspase3 monoclonal antibody (Caspase-3 immune staining, x400).

Table 3: Comparison of immunohistopathological parameters (VEGF, CD31, α - SMA, and Caspase 3) between all groups, Control group (GI), NAFLD group (GII) and Sorafenib group (GIII) :

Groups (n=15 for each)	VEGF %	CD31 %	α SMA %	Caspase 3 %
Control (GI)	0.00± 0.00	0.51± 0.01	0.20 ± 0.01	0.00 ± 0.00
NAFLD (GII)	15.046 ±0.69 **	16.67 ± 0.7 **	4.1 ± 0.19 **	3.67 ± 0.19 **
Sorafenib (GIII)	0.00 ± 0.00 ##	1.006 ± 0.075 ##	0.966 ± 0.07 ** ##	0.00 ± 0.00 ##

Result was expressed in the form of mean ± standard error. One way ANOVA with Turkey post hoc test. VEGF: vascular endothelial growth factor, CD31: Cluster of differentiation 31, α SMA: alpha smooth muscle actin. (%): it is ratio between areas that are positively stained in relation to all field areas. ** Significant (P ≤ 0.001), P: as compared with control rats. # Significant (P1 ≤ 0.001) P1: as compared with untreated NAFLD rats.

Discussion

Non-Alcoholic Fatty Liver Disease (NAFLD) is described as spectrum of diseases from simple steatosis to Non Alcoholic Steato-Hepatitis (NASH), fibrosis, cirrhosis, and finally it

may develop to hepatocellular carcinoma (HCC) (15). Angiogenesis is defined as formation of new blood vessels from pre-existing vessels, angiogenesis can occur in many pathological conditions in liver including progressive chronic liver disease (CLDs) and HCC. Pathological

angiogenesis occurring during the progression of CLDs as NAFLD is affected mainly by interactions between different hepatic cell populations especially Liver Sinusoidal Endothelial cells (LSECs) and Hepatic Stellate Cell (HSCs) together with several proangiogenic mediators as VEGF with its receptors that are expressed in LSECs and HSCs (5).

Capillarization of LSECs appears after liver injury leading to impaired oxygen diffusion from the sinusoids to the parenchyma, this hypoxic state provokes angiogenic process. Capillarized LSECs activates HSCs that induce collagen deposition leading to liver fibrosis, this also aggravates hypoxic state, this occurs together with VEGF (16). VEGF plays potent proangiogenic as well as profibrogenic roles (5).

There is a contradiction among the views on the role of angiogenesis in either resolution or aggravation of CLDs and NASH; *Elpek* (9) revealed that in CLDs; angiogenesis might contribute to the progression of disease during the wound healing process to maintain homeostasis in CLDs. Also, *Xi et al.* (10) suggested that suppression of angiogenesis could deteriorate fibrogenesis.

Another point of view was adopted by *De Leve* (17) who stated that HSCs activation plays a key role in liver fibrosis and contributes to overdeposition of extracellular matrix (ECM). Angiogenesis occurs before fibrogenesis and promotes HSC activation and fibrosis development, thus suppression of angiogenesis might be a potential target to counteract the progression of CLDs. However, *Adlia* (18) proved that targeted IFN α suppressed angiogenesis, but did not affect disease development. It suggested

that angiogenesis appeared in CLDs but did not affect fibrosis progression in the liver, and it needs further researches.

Therefore in the current study, we evaluated the development of angiogenesis in rats model of NAFLD and tried to determine the possible protective effects of antiangiogenic therapy (sorafenib) in preventing the progression of NAFLD. Our study was conducted on adult male Albino rats was induced by feeding high fat, high fructose diet (HFD and HFr) as a model for NAFLD. The combination of fat and fructose exacerbates hepatic steatosis and intensified NAFLD progression (19).

Fructose is a lipogenic, pro-inflammatory dietary factor that results in oxidative stress and upregulation of TNF- α . It is metabolized by the liver producing purvate that acts as substrate for acetyl CoA which serves as the substrate for de novo lipogenesis(DNL) or acetyl CoAs are decarboxylated to malonyl CoA which inhibits mitochondrial β -oxidation (20). In addition, ip DEN was used because DEN is principally oxidized by cytochrome P-450 (CYPs) in the liver microsomes to metabolically bioactive metabolites forming reactive oxygen species (ROS). High intracellular levels of ROS can lead to damaged mitochondria (21).

Sorafenib was used in this study as antiangiogenic drug that acts as a multikinase inhibitors mainly tyrosine kinase inhibitor (TKI) of VEGF receptor (VEGFR2& VEGFR3), PDGF receptor and Raf/MAPK/ERK pathway thus preventing transcription of several proteins involved in angiogenesis process such as proliferation, differentiation, migration and survival of endothelium (8).

In the current study, our results showed a significant increase in triglycerides, cholesterol, and LDL in NAFLD group (GII) as compared with normal control group (GI). Also, HDL was decreased significantly in NAFLD group (GII) as compared with control group (GI) (**Table 1**) suggesting dyslipidemia which was found to have a positive association with NAFLD (22).

Delivery of excess fatty acids or DNL in the liver by excess mitochondrial acetyl CoA as a result of excess energy intake results in fatty acid esterification to cytoplasmic triglycerides, also, these TGs are packaged with apolipoprotein B100 (apo B100) by microsomal triglyceride transfer protein (MTTP) and are exported as VLDL particles causing dyslipidemia (23). Triglyceride rich lipoprotein remnants can be hydrolyzed by hepatic lipases producing small dense lipoprotein particles, as LDL-cholesterol particles among those with hypertriglyceridemia, this hypertriglyceridemia, and low HDL played a vital role in the pathogenesis of NAFLD (24).

Also, in this study, our results showed significant decrease in triglycerides, cholesterol, and LDL, and significant increase in HDL in treated group with oral sorafenib (GII) as compared to NAFLD diseased group (GI) (**Table1**) suggesting improvement of lipid profile that indicated alleviation and improvement of the metabolic function of liver in NAFLD (25). However, depending on our knowledge, there were not any previous studies reported the improvement of serum lipid profile with sorafenib use. But on the contrary *Verge et al.* (26) stated that TKIs do not induce hyperlipidemia.

Although, our findings may be explained by the role of mitochondria in β -oxidation of fatty

acids and citric acid cycle that results in decrease hepatic lipid accumulation. Activator of transcription called PGC-1 is the main regulator of mitochondrial oxidative phosphorylation and biogenesis. It is highly expressed in the liver, PGC-1 interacts with peroxisome proliferator activated receptor alpha (PPAR- α) thus elevating the expression of transcription factors as nuclear respiratory factor-1 and mitochondrial transcription factor A, which in turn are responsible for high synthesis of new mitochondria, therefore, the number of mitochondria and their oxidative capacity by mitochondrial β -oxidation of fatty acids markedly increased. It was observed that PGC1 α expression decreased during NASH with fibrosis. Interestingly, sorafenib restored PGC1 α expression, frequently; mitochondrial citric acid cycle and oxidative capacity were restored with sorafenib thus improving lipid profile (8).

In present study, the liver functions were assessed by measuring serum level of liver enzymes AST and ALT. The results showed a significant increase in the serum concentrations of AST and ALT in untreated NAFLD group in comparison with normal control group (**Table2**) suggesting liver injury. These results were in line with *Douglas et al.* (27). These findings were illustrated by effects of NAFLD in production of liver cell injury by oxidative stress and inflammation mediated by lipotoxicity (steatohepatitis) (27).

In this work, oral administration of sorafenib in NAFLD induced rats produced significant decreased in AST and ALT relative to untreated NAFLD group (**Table2**). This suggested the improvement of liver functions and decrease

the risk for liver injury as any type of liver cell injury can reasonably increase liver enzymes (28). These results may be explained by the ability of sorafenib to decrease lipotoxicity and inflammatory state, thus it can improve hepatic cell condition by its mild anti-inflammatory effect and improvement of mitochondrial integrity in small dose use in NASH model (8).

In the present study, it had been found that the serum albumin was decreased significantly in untreated NAFLD group as compared with normal control group (Table 2) suggesting deterioration in synthetic function of liver. These results were in agreement with *Hadizadeh et al.* (29). Also, sorafenib treated rats showed a significant increase in the serum level of albumin when compared with untreated NAFLD group (Table 2) suggesting improvement of synthetic function of liver in accordance with *Bernardi et al.* (30).

This can be explained by capacity of liver microsomes to incorporate amino acids is depressed, causing a generalized loss of the ability of liver to synthesize albumin. The lowered level of serum albumin indicates increasing of hepatocellular damage accompanied with NASH and hepatic fibrosis (29). Also, decrease albumin level can be helped by DEN intoxication which causes disruption and disassociation of polyribosomes on endoplasmic reticulum and thereby reduces the biosynthesis of protein (31). It was found that the severity of NAFLD was negatively correlated with albumin level. Sorafenib treatment reduces inflammatory state in NASH or hepatic fibrosis, frequently increasing albumin (32).

In the current study, there was a significant decrease in hepatic GSH, one of the

most important antioxidants, in untreated NAFLD group when compared with normal control group (Table 2) suggesting development of oxidative stress in NAFLD. These results were in accordance with *Wensheng et al.* (33). This finding denoted that GSH depletion plays an important part in the context of NAFLD. The depletion of GSH could be due to decreased uptake by the mitochondria because of increased cholesterol within mitochondrial membrane and/or decreased synthesis of S-adenosyl methionine, the precursor of GSH. Also, it was suggested that GSH might be utilized and oxidized to GSSG in order to cope with increased oxidative stress, therefore explaining the GSH depletion (34).

In the current work, sorafenib treated group showed significant increase in the tissue level of GSH when compared with untreated NAFLD group (Table 2) suggesting relief of oxidative stress, decrease ROS prevents depletion of antioxidant molecules such as GSH (33). Although, depending on our knowledge, there were not any previous studies illustrated the rise of GSH with sorafenib treatment in fat laden hepatic parenchyma. On the contrary, sorafenib could induce the generation of ROS in tumor cell in vivo and vitro in dose dependent manner with high dose to accelerate death of abnormal tumor cells. However, our findings about increased GSH with sorafenib could be explained by the role of sorafenib in prevention mitochondrial dysfunction and improvement of mitochondrial respiration, mitochondrial synthesis with NAFLD (8).

The biochemical results were further supported by histopathological examinations of the liver parenchyma (figure 1). Liver specimens in NAFLD rats showed significant steatosis with

damaged lobular structures, blurred boundaries between liver cells, coagulative necrosis (grade3) (**figure 1b**), this is in line with *Song et al. (25)*. In the present study, there was apparent decline in fatty infiltration in sorafenib treated group with decreasing the size and number of lipid droplet “simple steatosis, grade1” (**Figure 1c**). This suggested improvement that was described by *Takahashi and Fukusato (14)* who stated angiogenesis and adipogenesis are coupled processes.

Regarding immunohistopathological examination for liver specimens with image analysis for reaction against VEGF antibodies in control (GI) and NAFLD (GII) ;there were strong reactions against VEGF monoclonal antibody in untreated NAFLD rats (**figure 2b**) when compared with control group (**figure 2a**). These findings were confirmed by significant increase in % area affected in image analysis (**table 3**), this suggested the presence of excessive angiogenesis within hepatic parenchyma in NAFLD rats. These results were in agreement with *Guo et al. (35)* who confirmed that increase VEGF expression had strongly appeared in areas with extended fatty infiltration.

However in the current work, sorafenib treated rats showed absence of the immunohistological expression of VEGF against VEGF antibody (**figure 2c**) when compared with untreated NAFLD rats (**figure 2b**) suggesting relief of angiogenic state under sorafenib use. These results were in agreement with *Berretta et al. (36)*. Sorafenib is antiangiogenic agent by inhibiting the TK activities of VEGFR 2, 3 and of PDGFR. Moreover, it also inhibits RAF kinases which are members of serine/threonine-specific

protein kinases family, frequently prevent transcription of several proteins involved in angiogenesis process such as proliferation, differentiation, migration and survival of endothelium (8).

In the current study, referring to immunohistopathological analysis in liver biopsy for reaction against CD31 antibodies in normal group (GI), and NAFLD (GII); there were strong reactions against CD31 antibodies in untreated NAFLD group (**figure 3b**). These findings were confirmed by significant increase in % immunoexpressed areas in image analysis (**table 3**) when they were compared with very weak reactions in control one (**figure 3a**) suggesting increase microvascular density in the liver tissue, these results were in agreement with *Li et al. (37)*. However, sorafenib treated rats (GIII) decreased the expression of CD31 antibodies (**figure 3c**). These findings were confirmed by significant decrease in CD31 immunoexpression by image analysis (**Table 3**) in comparison with untreated NAFLD rats (**figure 3b, table 3**) suggesting distinct reduction of vascular density with sorafenib treatment. These results were in harmony with *Muselaers et al. (38)*.

The reduction of CD31 expression indicates prevention of neovascularization process as suggested by *Li et al. (37)*. These can be explained by identification of role of CD31 differentiation antigen, as integral membrane protein, it is expressed on the surface of platelets and endothelial cells (ECs). It supports the integrity of endothelial cell-cell junction. Therefore, it resists mechanical force under conditions of fluid shear stress. The role CD31 in angiogenesis was approved (37).

In the present study, immunohistopathological examination with performing image analysis for reaction against α SMA antibodies in NAFLD group (GII) showed that there were strong staining reactions against α SMA monoclonal antibodies abnormally within hepatic parenchyma near the sites of fatty infiltration (**figure 4b**) in comparison with normal control group (**figure 4a**). These results were confirmed by significant increase in % immune staining area against α SMA antibodies via image analysis (**table3**) denoting high amount of α -SMA protein expression with untreated NAFLD group. These findings were in agreement with *Inoue et al. (39)*.

However, immune expression reaction against α SMA antibodies in treated group (GIII) showed decrease in α -SMA- positive areas within hepatic parenchyma in which staining reactions against α SMA monoclonal antibodies were restricted to site of hepatic vessels walls (**figure 4c**) with significant decrease in % immune staining areas against α SMA antibodies by image analysis in sorafenib treated group (**table 3**) when compared with NAFLD group, this suggested possible role of sorafenib in preventing liver fibrosis. These findings were in harmony with *Stefano et al. (8)*.

These results were illustrated by proinflammatory cytokines with disease progression play a vital role in HSC activation and differentiation to myofibroblast with increase fibrosis and α -SMA expression (**39**). Also, sorafenib decreases the number of activated HSCs. Therefore, it reduces collagen deposition and prevents fibrosis development with decreasing α -SMA expression (**8**).

In the present work, there were strong immune reactions against Caspase-3 monoclonal antibodies in hepatic parenchyma “Caspase-3 immunolabeled hepatocytes” observed around areas of steatosis (**figure5b**), with significant increase in % immune staining area with image analysis in untreated NAFLD group (**Table3**) when compared with control rats (GI) which had no staining reaction or expression in image analysis (**figure 5a & table3**) suggesting occurrence of apoptosis in NAFLD rats. These results were in consistent with *Mohamed and Magdy (40)*.

These findings were explained by liptotoxicity with subsequent ROS production in NAFLD result in inflammation ending in cell death and fibrosis. ROS cause nuclear translocation of Nuclear Factor Kappa B (NF- κ B) translocation into the nucleus. NF- κ B induces apoptotic cell death by inducing transcriptional expression of the Fatty Acid Synthetase (FAS) ligand that was normally repressed. FAS ligand binds to FAS on adjacent hepatocytes and leads to caspase 9 activation with subsequent downstreaming other caspases including caspase3-activation which promotes apoptosis of hepatocytes (**41**).

Regarding the immune reaction against Caspase 3 antibodies in NAFLD groups (GII) and sorafenib groups (GIII), there were no reactions against caspase3 monoclonal antibodies in sorafenib treated rats (**figure 5c**). These were confirmed by absence of immunostaining cells in image analysis in sorafenib groups (GIII) (**table 3**), when compared with untreated NAFLD rats (**figure 5b & table 3**) suggesting relief of apoptosis. These results were in line with

Thapaliya et al. (42). These results were illustrated by the beneficial anti-inflammatory effect of sorafenib. Oxidative stress and cytokine production triggering cell death, inflammation can be possibly adjusted by the potent effect of sorafenib in correction of mitochondrial dysfunction, mainly by enhancing PGC1 α expression (8).

There are very limited researches about information of angiogenesis markers in NAFLD. In our study, we used only the immunoexpression of VEGF and CD31 as 2 markers for hepatic angiogenesis. Also, we analyzed only reduced glutathione as antioxidative stress marker without evaluation of oxidative stress markers. Therefore, further researches should evaluate other angiogenic markers with NAFLD together with oxidative stress markers analysis in hepatic tissue for accurate assessment of NAFLD prognosis with angiogenesis development.

We used sorafenib drug that has a strong anti-angiogenic action as it could inhibit several pathways involved in angiogenic process (8). Thus, this helped us to estimate the crucial role of angiogenesis suppression in prevention of NAFLD progression by the use of several biochemical, histopathological and immunohistopathological examination. Also, We evaluated the occurrence of apoptosis of hepatocytes by immunoexpression of caspase3 and appearance of hepatic fibrosis by α -SMA, these markers allowed us to follow up of NAFLD progression.

In conclusion: Angiogenesis revealed another pathological mechanism accelerating the NAFLD progression to liver fibrosis. This was confirmed

by histopathological, and immunohistochemical examination. Antiangiogenic agent (sorafenib) suggested as effective preventing therapy that restrains NAFLDs progression by inhibiting angiogenic process.

ACKNOWLEDGMENT

This study was supported by Dr. Abdel Hady Shebl, lecturer of Pathology, Faculty of Medicine, Mansoura University.

References:

- 1) **Bettermann, K., Hohensee, T., & Haybaeck, J.:** Steatosis and steatohepatitis: complex disorders. *Int J Mol Sci*; 15(6), 9924-9944, 2014.
- 2) **Takahashi, Y., Sugimoto, K., Inui, H., & Fukusato, T.:** Current pharmacological therapies for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World J Gastroenterol* , 21(13), 3777-3785, 2015.
- 3) **Mederacke, I., Hsu, C.C., Troeger, J.S., Huebener, P., Mu, X., Dapito, D.H., et al. :** Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. *Nature Communication*, 4, 2823, 2013.
- 4) **Boghdadia, I.M., Koraha, T.E., Dalaa, A.G., & Oafb, O.I.:**Angiogenesis in liver cirrhosis. *Menoufia Medical Journal*, 30(1), 971-1262, 2017.
- 5) **Sahin, H., Borkham-Kamphorst, E., Kuppe, C., Zaldivar, M., Grouls, C., & Al-samman, M.:** Chemokine Cxcl9 Attenuates Liver Fibrosis-Associated Angiogenesis in Mice. *HEPATOLOGY*, 55 (5), 1610- 1619, 2012.
- 6) **Ding, B., Cao, Z., Lis, R., Nolan, D.J., Guo, P., Simons, M., et al.:** Divergent angiocrine signals from vascular niche balance liver

regeneration and fibrosis. *Researcher letter*, 505, 97-102, 2014.

7) **Zhao, Z.M., Liu, H.L., Sun, X., Guo, T., Shen, L., Tao, Y.Y. and Cheng-Hai Liu, C.H.:** Levistilide A inhibits angiogenesis in liver fibrosis via vascular endothelial growth factor signaling pathway. *Experimental Biology and Medicine*, 242(9), 974–985, 2017.

8) **Stefano, J.T., Pereira, I.V., Torres, M.M., Bida, P.M., Coelho, A.M., & Xerfan, M.P. :** Sorafenib prevents liver fibrosis in a non-alcoholic steatohepatitis (NASH) rodent model. *Brazilian Journal of Medical and Biological Research*; 48(5), 408-414, 2015.

9) **Elpek, G.O.:** Angiogenesis and liver fibrosis. *World J Hepatol.*, 7(3), 377–391, 2015.

10) **Xi, L., Yao, Q., Liu, H., Jin, Q., Xu, B., Zhang, S., & Tu, C.:** Placenta growth factor silencing ameliorates liver fibrosis and angiogenesis and inhibits activation of hepatic stellate cells in a murine model of chronic liver disease. *J. Cell. Mol. Med.*, 21(10), 2370-2385, 2017.

11) **Poudyal, H., Campbell, F., & Brown, L. :** Olive Leaf Extract Attenuates Cardiac, Hepatic, and Metabolic Changes in High Carbohydrate–, High Fat–Fed Rats. *J. Nutr.* 140; 946–953, 2010.

12) **Li, X., Zhou, X.P., Guan, Y.S., & Wang, Y.X.:** Magnetic resonance imaging of hepatocellular carcinoma induced by diethylnitrosamine in Sprague Dawley rats. *hepatobiliary and pancreatic diseases international*, 4(3), 427-432, 2005.

13) **Bancroft, J.P., Stevenes, A., & Turner, D.R. :** *Theory and Practice of Histological Techniques* (3rd ed), (pp. 1-726). Clurehill Livingston, Edinburgh, London, 1990.

14) **Takahashi., Y., & Fukusato T.:** Histopathology of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World J Gastroenterol.*, 20(42), 15539–15548, 2014.

15) **Kanwar, P., & Kowdley, K.V. :** The Metabolic Syndrome and Its Influence on Nonalcoholic Steatohepatitis. *Clin Liver Dis.*, 20(2), 225-243, 2016.

16) **Miyao, M., Kotani, H., Ishida, T., Kawai, C., Manabe, S., Abiru, H., et al. :** Pivotal role of liver sinusoidal endothelial cells in NAFLD/NASH progression. *Lab Invest*, 95, 1130–1144, 2015.

17) **DeLeve, L.D.:** Liver sinusoidal endothelial cells in hepatic fibrosis. *Hepatology*, 61, 1740–1746, 2015.

18) **Adlia A.:** Angiogenesis in liver fibrosis. *Groningen*, 1-133, 2017.

19) **Jensen, V.S., Hvid, H., Damgaard, J., Nygaard, H., Ingvorsen, C., Wulff, E., et al. :** Dietary fat stimulates development of NAFLD more potently than dietary fructose in Sprague–Dawley rats. *Diabetol Metab Syndr.*, 10 (4), 2018.

20) **Dentin, R., Benhamed, F., Hainault, I., Fauveau, V., Foufelle, F., Dyck, J.R., et al.:** Liver-specific inhibition of ChREBP improves hepatic steatosis and insulin resistance in ob/ob mice. *Diabetes*, 55(8), 2159–2170, 2006.

21) **Tolba, R., Kraus, T., Liedtke, C., Schwarz, M., & Weiskirchen, R.:** Diethylnitrosamine (DEN)-induced carcinogenic liver injury in mice. *Lab Anim.*, 49(1), 59-69, 2015.

22) **Peng, K., Mo, Z, & Tian, G.:** Serum Lipid Abnormalities and Nonalcoholic Fatty Liver Disease in Adult Males. *Am J Med Sci.*, 353(3), 236-241, 2017.

23) **Stanhope, K. L., Schwarz, J.M., Keim, N.L., Griffen, S.C., Bremer, A.A., Graham, J.L., et**

- al.*: Consuming fructose-, not glucose-sweetened beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. *J. Clin. Invest.* 119, 1322–1334, 2009.
- 24) **Du, T., Sun, X., & Yu, X.** : Non-HDL cholesterol and LDL cholesterol in the dyslipidemic classification in patients with nonalcoholic fatty liver disease. *Lipids Health Dis*; 16-229, 2017.
- 25) **Song, L., Qu, D., Zhang, Q., jiang, J., Zhou, H., Jiang, R., et al.** : Phytosterol esters attenuate hepatic steatosis in rats with non-alcoholic fatty liver disease rats fed a high-fat diet . *Sci Rep.* , 7- 1604, 2017.
- 26) **Verge, B., Walter, T., and Cariou, B.**: ENDOCRINE SIDE EFFECTS OF ANTI-CANCER DRUGS; Effects of anti-cancer targeted therapies on lipid and glucose metabolism. *European Journal of Endocrinology*, 170, R43–R55, 2014.
- 27) **Douglas, R.L., Zaigham, A., Frank, A., Peter, F., Aamir G.K., Lee, G.K., et al.**: World Gastroenterology Organisation Global Guidelines: Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis. *Journal of Clinical Gastroenterology*, 48(6), 467–473, 2014.
- 28) **Gowda, S., Desai, P.B., Hull, V.V., Math, A.A., Vernekar, S.N., & Kulkarni, S.S.**: A review on laboratory liver function tests. *Pan Afr Med J.*, 3, 1-17, 2009.
- 29) **Hadizadeh, F., Faghihmani, E., & Adibi, P.**: Nonalcoholic fatty liver disease: Diagnostic biomarkers. *World J Gastrointest Pathophysiol.*, 8(2), 11–26, 2017.
- 30) **Bernardi, M. Maggioli, C., & Zaccherini, G.**: Human albumin in the management of complications of liver cirrhosis. *Crit Care.* 16(2), 1-211, 2012.
- 31) **Chen, G., Dai, Z.K., Liang, R.G., Xiao, S.J., He, S.Q., Zhao, H., et al.** : Characterization of diethylnitrosamine-induced liver carcinogenesis in Syrian golden hamsters. *Exp Ther Med.*, 3(2), 285–292, 2012.
- 32) **Mikolasevic, I., Racki, S.D., Zaputovic, L., Devcic, B., Jelic, I., & Lukenda, V.** : Relationship between non-alcoholic fatty liver disease and MIA syndrome. *Hemodialysis International*, 19 (3), 472–481, 2015.
- 33) **Wensheng, L., Baker, R., Baker, S., & Zhu, L.**: Antioxidant Mechanisms in Nonalcoholic Fatty Liver Disease. *Current drug targets*, 16(12), 1301-1314, 2015.
- 34) **Cheng, S.B., Liu, H.T., Chen, S.Y., Lin, P.T., Lai, C.Y., & Huang, Y.C.** : Changes of Oxidative Stress, Glutathione, and Its Dependent Antioxidant Enzyme Activities in Patients with Hepatocellular Carcinoma before and after Tumor Resection. *PLoS One.*, 12(1), e0170016, 2017.
- 35) **Guo, D., Wang, Q., Li, C., Wang, Y., & Chen, X.**: VEGF stimulated the angiogenesis by promoting the mitochondrial functions. *Oncotarget.*, 8(44), 77020–77027, 2017.
- 36) **Berretta, M., Luca Rinaldi, L., Benedetto, F.D., Arben Lleshi, A., Re, V.D., Gaetano Facchini, G., et al.** : Angiogenesis Inhibitors for the Treatment of Hepatocellular Carcinoma. *Front Pharmacol*, 7, 1-428, 2016.
- 37) **Li, X., Yao, Q., Liu, H.C., Jin, Q.W., Xu, B.L., Zhang, & Tu, C.T.**: Placental growth factor silencing ameliorates liver fibrosis and angiogenesis and inhibits activation of hepatic stellate cells in a murine model of chronic liver

disease. *J. Cell. Mol. Med.*, 21 (10), 2370-2385, 2017.

38) Muselaers, C.H., Stillebroer, A.B., Desar, I.M.E., Boers-Sonderen, M.J., van Herpen, C.M., Weijert, M.C., & et al. : Tyrosine Kinase Inhibitor Sorafenib Decreases ¹¹¹In-Girentuximab Uptake in Patients with Clear Cell Renal Cell Carcinoma. *J Nucl Med.*, 55 (2), 242-247, 2014.

39) Inoue, A., Obayashi, K., Sonoda, Y., Nakamura, A., Ueno, T., Kuhara, S., et al.: Regulation of matrix metalloproteinase-1 and alpha-smooth muscle actin expression by interleukin-1 alpha and tumour necrosis factor alpha in hepatic stellate cells. *Cytotechnology*, 69(3), 461–468, 2017.

40) Mohamed, A.K., & Magdy, M.: Caspase 3 role and immunohisto-chemical expression in assessment of apoptosis as a feature of H1N1 vaccine-caused Drug-Induced Liver Injury (DILI). *Electron Physician.*, 9(5), 4261–4273, 2017.

41) Patel, V., & Sanyal, A.J. : Drug-Induced Steatohepatitis. *Clin Liver Dis.*, 17(4), 533vii., 2013.

42) Thapaliya, S., Wree, A., Povero, D., Inzaugarat, M.E., Berk, M., Dixon, L., et al.: Caspase 3 inactivation protects against hepatic cell death and ameliorates fibrogenesis in a diet induced NASH model. *Dig Dis Sci.*, 59(6), 1197–1206., 2014.