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Study of the effects of some oral Anti-diabetic drugs and Silymarin on Experimentally Induced Non-alcoholic Fatty Liver in Rats

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Abstract: Nonalcoholic fatty liver disease (NAFLD) is a chronic disease which has a wide spectrum of liver-related histological damage and becoming a global epidemic. Predisposing factors for NAFLD and related complications include insulin resistance and type 2 diabetes mellitus. Therefore, intervention with anti-diabetic medications may prevent and delay the development of NAFLD or have a therapeutic application. The focus of this study is to evaluate the evidence supporting the efficacy of anti-diabetic medications and silymarin in the treatment of NAFLD. Methods: 48 male albino rats divided in to 4 groups: Group I received standard diet, Group II received a high fat diet (HFD), Group III treated with empagliflozin (10 mg/ kg/ day) and Group IV treated with silymarin (100 mg/ kg/ day). After 5 weeks rats were scarified and serum (ALT, AST, TG, cholesterol, random blood glucose and insulin level), hepatic (MDA, GSH, TG and TGF-β) and body weight were measured. Histopathological examination was done. Results: empagliflozin, and silymarin had good results in ameliorating NAFLD as regard anti-inflammatory, anti-hyperlipidemic, anti-fibrotic effects and decreasing hepatic lipid content. Clinically such findings may suggest that, these drugs could be used in treatment of NAFLD patients especially those have type 2 DM and liver fibrosis.
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Keywords: NAFLD; NASH; anti-diabetic medication; silymarin.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver disease currently worldwide. NAFLD is a major cause of chronic liver disease. Fatty liver ranges from simple triglyceride accumulation (steatosis), non-alcoholic steato-hepatitis (NASH) to the most serious, cirrhosis and also hepatocellular carcinoma. It is reported that almost 10-20% of individuals with NAFLD have NASH and 10-15% of individuals with NASH develop cirrhosis **(Alam et al., 2018).**

The pathogenesis of NAFLD is complex, involving insulin resistance, oxidative stress, lipid peroxidation and mitochondrial dysfunction. Insulin resistance is the most important pathogenic factor for the development of NAFLD **(Kuchay et al., 2018).**

High-fat diet is the commonest cause of obesity in the world. Diet-induced weight loss and life style modifications, including physical exercise and qualitative changes in the diet, have significant effects on NAFLD and antagonizes insulin resistance (IR). However, only a small percentage of patients with NAFLD can apply these measures efficiently **(Souza et al., 2010).**

Empagliflozin is a potent oral anti-diabetic drug that inhibits sodium-glucose co-transporter 2 (SGLT-2). By inhibiting this transporter, empagliflozin promotes urinary glucose excretion, resulting in decrease in blood levels of glucose and improves insulin resistance in patients with type 2 diabetes leading to down-regulation of carbohydrate-responsive element-binding protein (ChREBP), a transcription factor responsible for activating the machinery for fatty acid synthesis. Improvement in insulin resistance (hyperinsulinemia) results in down-regulation of SREBP-1c and the blockage of hepatic lipogenesis **(Kuchay et al., 2018).**

Silymarin has many hepato-protective properties such as anti-inflammatory, anti-proliferative, immune-modulatory and anti-cholesterolemic characters. So, silymarin has been used in the treatment of many liver disorders **(Marin et al., 2017).**

2. Material and Methods:

❖ **Animal grouping and design of the work:**

48 male albino rats each weighing 100-120 grams were kept under similar housing conditions and were divided in to 4 groups as the following:

- **Group I (serving as negative control group):**

12 rats were fed with standard diet and received normal saline at a dose of 1ml/ rat by gastric tube daily throughout the study, and served as normal control group.

- **Group II (serving as NAFLD control group):**

12 rats were fed a high fat diet (HFD); which consists of the standard crushed rat chow diet, 10% animal fat (sheep tallow) and 2% cholesterol for 16 weeks **(Wang et al., 2013).**

- **Group III (Empagliflozin group):**

12 rats were fed HFD for 8 weeks then during the next 8 weeks of the study the rats received HFD plus empagliflozin suspension, at a daily dose of 10 mg/ kg (1.6 ml) by gastric tube **(Jojima et al., 2016).**

- **Group IV (Silymarin group):**

12 rats were fed HFD for 8 weeks then during the next 8 weeks of the study the rats received HFD plus silymarin suspension, at a daily dose of 100 mg/kg (1.4 ml) by gastric tube **(Huang et al., 2010).**

❖ **Outcome measures:**

1- Biochemical studies:

(A) Collection of blood samples:

Blood samples were collected from the retro-orbital venous plexus of rat eye by using heparinized capillary tubes. The collected blood was then centrifuged at 3000 round/minute for 30 minutes. Then the serum was transferred into clean vials and stored at -18°C for biochemical parameters determination and the abdomens of the rats were dissected and the livers were excised to measure the following parameters:

(B) Biochemical measurements:

➢ **Serum parameters:**

- Alanine aminotransferase (ALT).
- Aspartate aminotransferase (AST).
- Triglycerides (TG).
- Cholesterol.
- Random blood glucose.
- Serum insulin level.

➢ **Liver homogenate parameters:**

Liver samples were homogenized for measurement of:

- Hepatic levels of malondialdehyde (MDA).
- Hepatic levels of reduced glutathione (GSH).
- Liver tissue content of triglycerides.
- Transforming growth factor β (TGF-β).

2- Body weight recordings:

Body weight was recorded to the rats of all groups at the beginning and at the end of the study.

3- Histopathology:

A small piece of liver was immediately fixed in 10% neutral buffered formalin then embedded in paraffin wax. For light microscopic analysis of liver histology, the paraffin-embedded liver tissues were cut into 4µm sections, and standard hematoxylin-eosin (H & E) staining was performed. Hepatic fibrosis was assessed by Masson trichrome stain.

The liver sections were scored according to the NAFLD Activity Score (NAS), which includes the features of active liver injury.

The score is defined as the sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2); thus ranging from 0 to 8 **(Kleiner et al., 2005).**

Ten light microscopic fields were viewed on each section and scored for the severity of hepatic steatosis, inflammation, ballooning and fibrosis according to the following criteria:

➢ **For hepatic steatosis**:

Score 0: no fat.

Score 1: steatosis occupying less than 33% of the hepatic parenchyma.

Score 2: 34–66% of the hepatic parenchyma.

Score 3: more than 66% of the hepatic parenchyma.

➢ **For inflammatory cell infiltration**:

Score 0: none.

Score 1: 1-2 foci/field.

Score 2: 3-4 foci/field.

Score 3: more than 4 foci/field.

➢ **Hepatocellular ballooning**

Score 1: few ballooned cells.

Score 2: marked ballooning.

➢ The staging of **hepatic fibrosis** was investigated by Masson staining as:

Stage 1: Perisinusoidal / pericellular fibrosis; focally or extensively present (lesion in the central vein area).

Stage 2: Zone 3 perisinusoidal/ pericellular fibrosis with focal or extensive periportal fibrosis (lesion in the central vein area and expansion to the surrounding area).

Stage 3: Portal fibrosis with focal or extensive bridging fibrosis.

Stage 4: Cirrhosis **(Brunt et al., 1999; Kleiner et al, 2005**).

3. Results

The effects of treatment of NAFLD rats with empagliflozin (10 mg/kg, p.o. /day) and silymarin (100 mg/kg, p.o. /day) together with high fat diet (HFD) for 8 weeks preceded by 8 weeks during which the rats were receiving HFD only on Serum ALT, AST, total cholesterol, serum TG and random blood glucose levels:

High fat diet (group II) significantly increased serum levels of ALT by (490.15 %), AST by (533.69 %), total cholesterol by (103.08 %), serum TG by (95.49 %) and random blood glucose by (157.49%) compared to normal control group (group I).

Empagliflozin 10 mg/ kg (group III) administration significantly decreased serum levels of ALT by (54.77 %), AST by (29.83 %), total cholesterol by (38.11 %), serum TG by (32.51 %) and random blood glucose by (33.61%) compared to HFD group (group II).

Silymarin 100 mg/ kg (group IV) administration significantly decreased serum levels of ALT by (61.06 %), AST by (36.81 %), total cholesterol by (21.61 %), serum TG by (12.46 %) and random blood glucose by (6.7 %) compared to HFD group (group II).

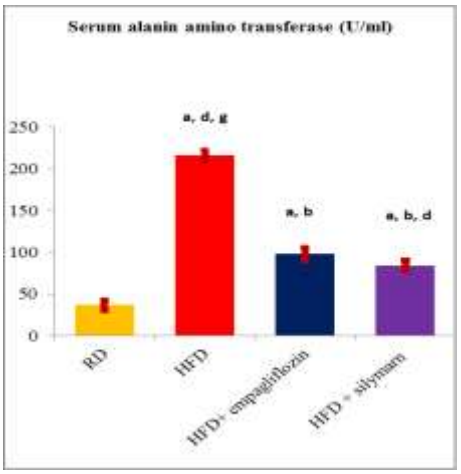


Figure (1): The effects of different studied groups on serum alanine aminotransferase (ALT) (U/ml):

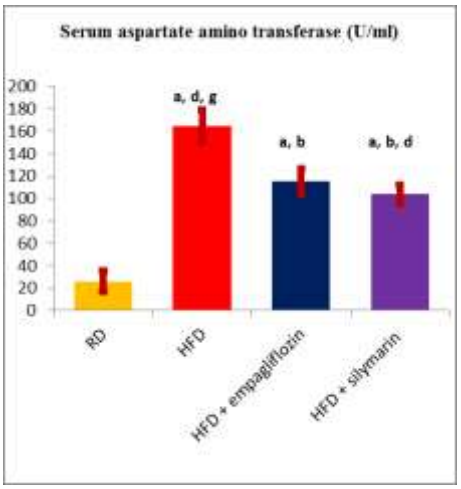


Figure (2): The effects of different studied groups on serum aspartate amino transferase (AST) (U/ml):

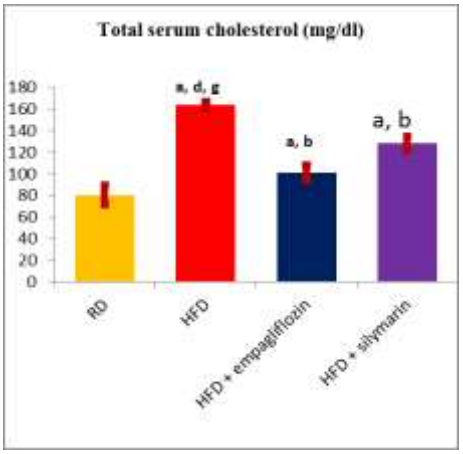


Figure (3): The effects of different studied groups on total serum cholesterol (mg/ dl):

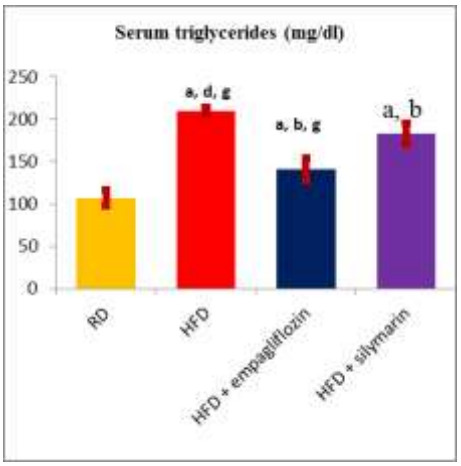


Figure (4): The effects of different studied groups on serum triglycerides (TG) (mg/ dl):

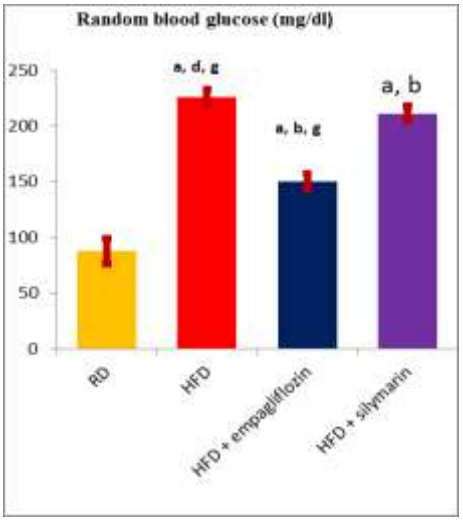


Figure (5): The effects of different studied groups on random blood glucose (mg/ dl):

a: Significantly different from negative control group.

b: Significantly different from HFD group.
d: Significantly different from HFD+ empagliflozin 10 mg/kg / day treated group.
g: Significantly different from HFD+ silymarin 100 mg/kg / day treated group.

2- The effects of treatment of NAFLD rats with empagliflozin (10 mg/kg, p.o. /day), and silymarin (100 mg/kg, p.o. /day) together with high fat diet HFD for 8 weeks preceded by 8 weeks during which the rats were receiving HFD only on hepatic level of MDA, GSH, TG and TGF-β:

High fat diet (group II) administration significantly increased hepatic levels of MDA by (103.43 %), decreased GSH by (45.3 %), increased hepatic TG by (231.1 %) and TGF- β by (601.68 %) compared to normal control group (group I).

Empagliflozin 10 mg/ kg (group III) administration significantly decreased hepatic levels of MDA by (42.42 %), increased GSH by (32.01 %), deceased TG by (52.77 %) and TGF- β by (30.28 %) compared to HFD group (group II).

Silymarin 100 mg/ kg (group IV) administration significantly decreased hepatic levels of MDA by (44.60 %), increased GSH by (41.58 %), deceased TG by (41.95 %) and TGF- β by (41.3 %) compared to HFD group (group II).

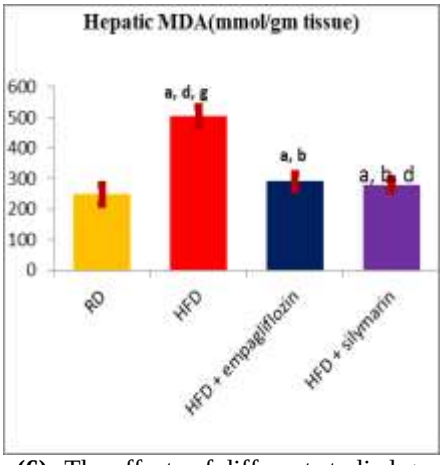


Figure (6): The effects of different studied groups on hepatic malondialdhyde (MDA) (mmol/ gm tissue):

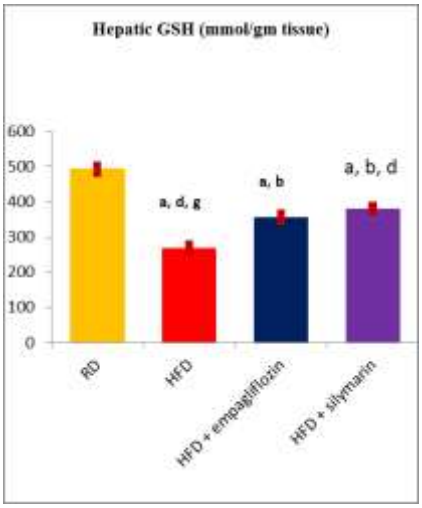


Figure (7): The effects of different studied groups on hepatic reduced glutathione (GSH) (mmol/ gm tissue):

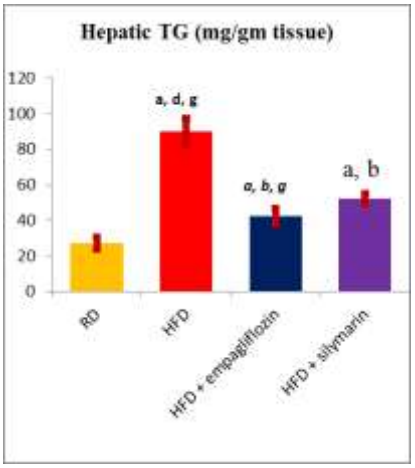


Figure (8): The effects of different studied groups on hepatic triglycerides (TG) (mg/ gm tissue):

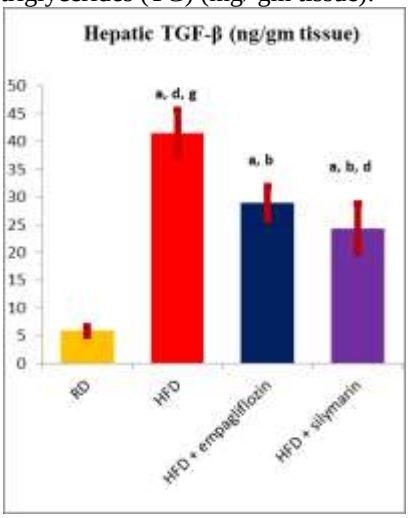


Figure (9): The effects of different studied groups on hepatic transforming growth factor-β (TGF-β) (mg/ dl):
a: Significantly different from negative control group.
b: Significantly different from HFD group.
d: Significantly different from HFD+ empagliflozin 10 mg/kg / day treated group.
g: Significantly different from HFD+ silymarin 100 mg/kg / day treated group.

The effects of treatment of NAFLD rats with empagliflozin (10 mg/kg, p.o. /day) and silymarin (100 mg/kg, p.o. /day) together with high fat diet HFD for 8 weeks preceded by 8 weeks of HFD only on body weight, Serum insulin level and NASH Score.

High fat diet (group II) significantly increased body weight by (18.14 %), serum insulin level by (156.72 %) and NASH Score by (377.64 %) compared to normal control group (group I).

Empagliflozin 10 mg/ kg (group III) administration significantly decreased body weight by (9.78 %), serum insulin level by (30.35 %) and NASH Score by (43.59 %) compared to HFD group (group II).

Silymarin 100 mg/ kg (group IV) administration non-significantly decreased body weight by (1.41 %), significantly decreased serum insulin level by (19.68 %) and NASH Score by (14.16 %) compared to HFD group (group II).

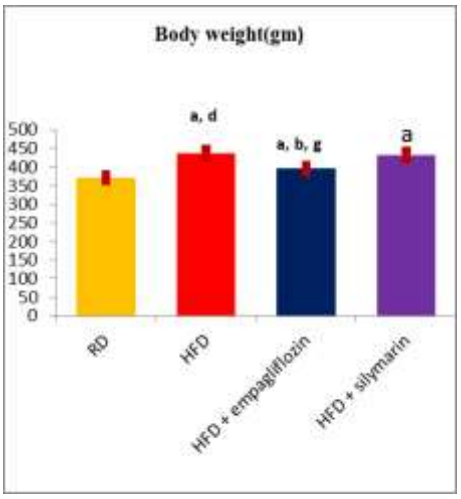


Figure (10): The effects of different studied groups on body weight (gm):

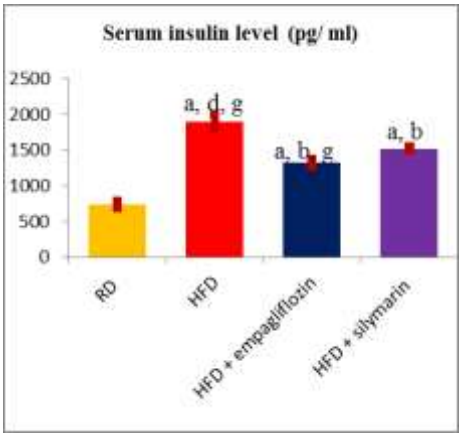


Figure (11): The effects of different studied groups on serum insulin (pg/ ml):

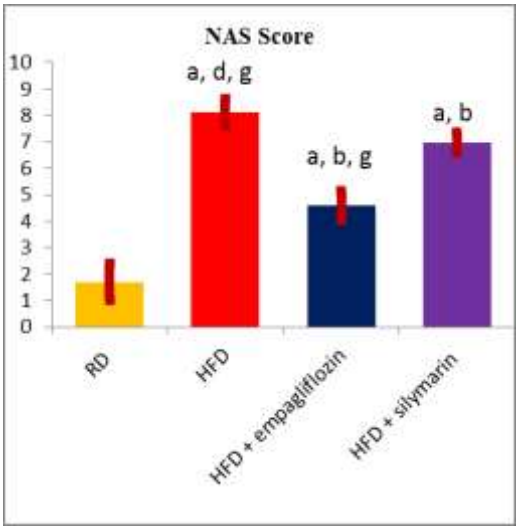


Figure (12): The effects of different studied groups on NAS score:
a: Significantly different from negative control group.
b: Significantly different from HFD group.
d: Significantly different from HFD+ empagliflozin 10 mg/kg / day treated group.
g: Significantly different from HFD+ silymarin 100 mg/kg / day treated group.

Histopathological findings

Group I (Negative control group): the pathological study of this group confirmed the clinical serological parameters. Liver section of this group showed normal structure and architecture (Haematoxylin & Eosin ×100).

Group II (High fat diet group): liver section of HFD fed group II showed severe degree of micro and macrovescicular steatosis and severe hepatocellular ballooning with frequent foci of inflammatory cells (Haematoxylin & Eosin ×200). Liver section of HFD fed group II showed marked portal fibrosis (masson trichom stain×100).

Group III (Empagliflozin treated group): liver sections of rats treated with empagliflozin showed less frequent inflammatory foci and moderate steatosis (H & E x100). Liver sections of rats treated with empagliflozin showed mild perisinasoidal fibrosis (masson stain x100).

Group IV (Silymarin treated group): liver sections of rats treated with silymarin (group IX) showed marked steatosis and hydrobic changes together with inflammatory foci (H & E x100). Liver sections of rats treated with silymarin showed marked perisinasoidal fibrosis (masson stain x100).

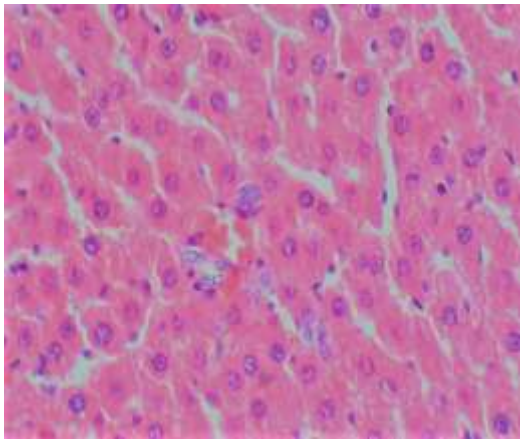
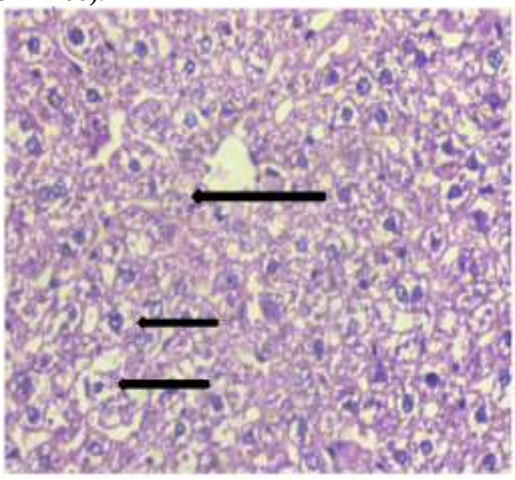
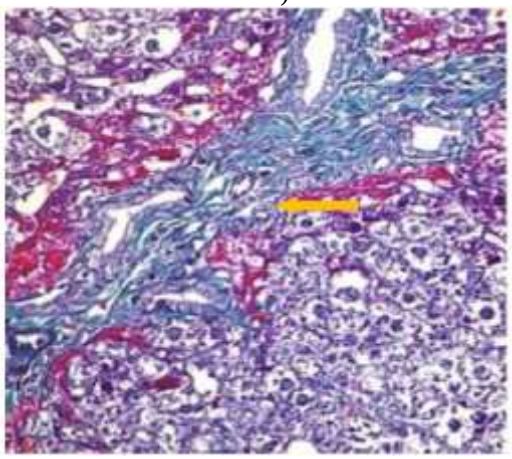


Figure (13): Liver section of control group I showing normal structure and architecture (Haematoxylin & Eosin ×100).

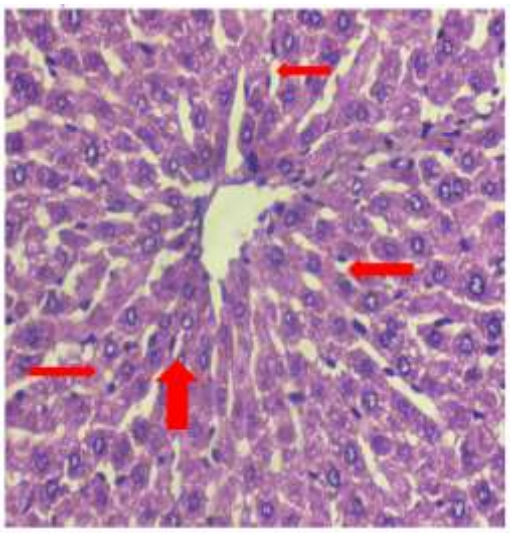


14-A)

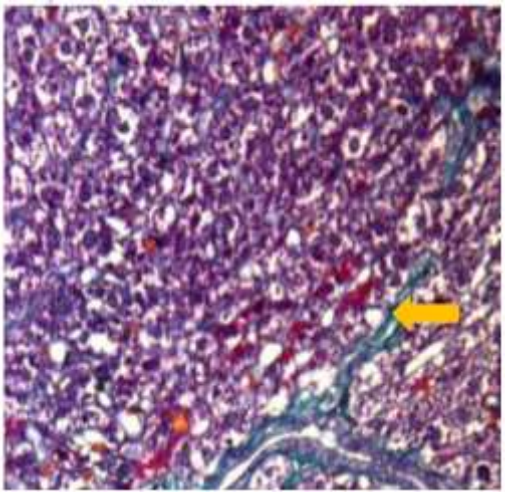


14-B)

Figure (14-A): liver section of HFD fed group II showing severe degree of micro and macrovescicular steatosis and severe hepatocellular ballooning with frequent foci of inflammatory cells (black arrow) (Haematoxylin & Eosin ×200). **Figure (14-B):** Liver section of HFD fed group III showing marked portal fibrosis (yellow arrow) (masson trichom stain×100).

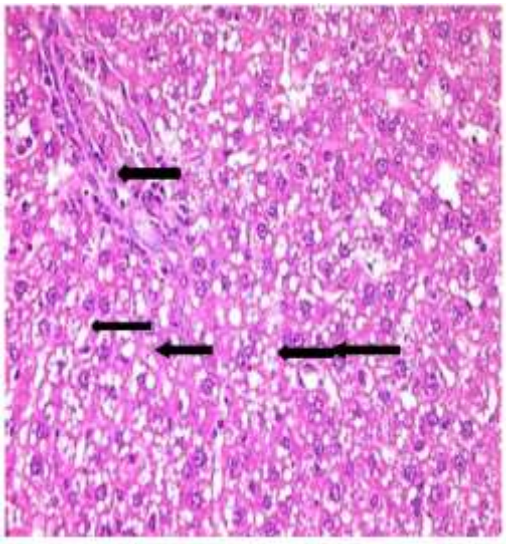


15-A)

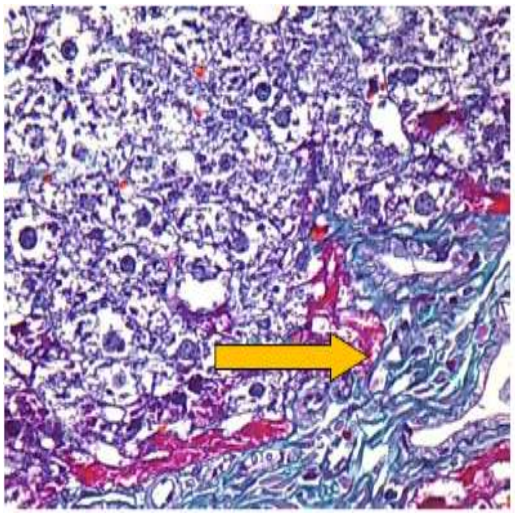


15-B)

Figure (15-A): Liver sections of rats treated with empagliflozin showing less frequent inflammatory foci and moderate steatosis (red arrows) (H & E x100). **Figure (15-B):** Liver sections of rats treated with empagliflozin showing mild perisinasoidal fibrosis (yellow arrow) (masson stain x100).



16-A)



16-B)

Figure (16-A): Liver sections of rats treated with silymarin (group IX) showing marked steatosis and hydrobic changes together with inflammatory foci (black arrows) (H & E x100). **Figure (16-B):** Liver sections of rats treated with silymarin showing marked perisinasoidal fibrosis (yellow arrow) (masson stain x100).

4. Discussion

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of liver disorders. It is a condition defined by the presence of steatosis in more than 5 % of hepatocytes **(Sanyal et al., 2011)** with a little or no alcohol consumption. NAFLD consists of the benign non-alcoholic fatty liver (NAFL), and the more severe non-alcoholic steato-hepatitis (NASH). NASH is a more progressive form of NAFLD and is characterized by steatosis, hepatocellular ballooning, lobular inflammation and almost always fibrosis **(Kleiner and Makhlouf, 2016).** In an effort to regenerate new cells,

The biological mechanism underlying steatosis occurrence and progression to NAFLD is not yet fully understood. Association of NAFLD with obesity, type 2 diabetes and metabolic syndrome suggests that; hyperglycemia, hyperlipidaemia, and insulin resistance are triggering factors in pathogenesis of NAFLD **(Mookhan et al., 2014).**

NAFLD can be considered as a hepatic manifestation of a metabolic syndrome. These findings have created a surge toward development of strategies to: control obesity, improve glycemic control, enhance insulin sensitivity and improve β-cell and hepatocyte function**.** Drug therapy has typically been focused on the management of associated risk factors such as diabetes, obesity, and hyperlipidemia, being predisposing factors for development of NAFLD **(Mookhan et al., 2014).**

Although a multiple animal models of NAFLD exist, high fat diet (HFD) administration has been a NASH progresses to cirrhosis **(Cobbina and Akhlaghi, 2017).**

NAFLD prevalence is high, being stated in around 20 to 30% of the general population in studies based on imaging methods. For histological studies, in selected groups of patients with risk factors for this disease, the prevalence may be higher, with steatosis found in 70% of obese patients and 35% of non-obese individuals, while NASH is seen in 18.5% of obese and 3% of non-obese patients. In patients with type 2 diabetes mellitus (DM2), the frequency of fatty liver disease can reach 75% **(Binobaid et al., 2018).**
widely used model and was chosen in the present study **(Asai et al., 2014).**

In the present study, HFD significantly increased serum levels of ALT, AST, total serum cholesterol, serum TG, random blood glucose, hepatic levels of MDA, decreased hepatic levels of GSH, increased hepatic TGs, TGF- β, serum insulin and NASH score compared to normal control group. These results agree **with Lin et al., 2011 and Yao et al., 2017.**

Sodium-glucose co-transporter-2 (SGLT2) inhibitors are a new class of oral antidiabetic drugs, which reduce hyperglycemia by promoting urinary glucose excretion independently of the secretion or action of insulin **(Tahrani et al., 2013).** Empagliflozin is a highly selective SGLT2 inhibitor that improves glycemic control in patients with T2DM **(Grempler et al., 2012).** In a preclinical model of obesity, empagliflozin triggered weight loss associated with reduction of body fat that was probably secondary to calorie wasting in the urine **(Vickers et al., 2014).**

Silymarin, extracted from the milk thistle, is a mixture of the three isomeric compounds silybin, silidianin, and silichristin **(Feher et al., 2008).** Some randomized controlled trials have shown beneficial effects of silymarin such as antioxidant therapy and improved survival against a variety of liver ailments **(Polyak et al., 2010).**

In the present study, administration of empagliflozin at a dose of 10 mg/ kg (group IV) ameliorated HFD induced NAFLD by significantly decreasing insulin resistance (decreased random blood glucose and serum insulin level), decreasing markers of acute liver injury (decreased serum ALT and AST) and decreasing serum and hepatic lipid content (total serum cholesterol, serumTG, hepatic TG and body weigh) and decreasing NAS score compared to HFD group (group II).

The results of the current study coincides with **Jojima et al., 2016,** whom studied the effects of empagliflozin 10 mg/ kg on steato-hepatitis in a novel mouse model of non-alcoholic steato-hepatitis and diabetes found that; empagliflozin significantly decreased serum serum levels of ALT, total cholesterol, TG, NAS score and body weight. They demonstrated that empagliflozin prevented the development of NASH in this model by its anti-inflammatory and anti-fibrotic effects. Expression of mRNA for FAS and ACC1, genes involved in fatty acid production (lipogenesis) were significantly lower in empagliflozin treated group than the vechile group. Empagliflozin decreased hepatic fibrosis by decreasing collagen deposition significantly compared to the vehicle group. Empagliflozin reduced hepatic inflammation, which was confirmed by decreased expression of TNF-α mRNAs significantly in the empagliflozin group compared with the vehicle group.

Another study agrees with these results done by **Xu et al., 2019,** whom documented that, after 8 weeks on a high-fat diet (HFD), mice exhibited obesity, accompanied by insulin resistance and low-grade chronic inflammation. Treatment with empagliflozin 0.01% (w/w) significantly decreased serum serum levels of ALT, AST, total cholesterol, TG, fasting glucose, insulin, NAS score and body weight. They explained their results by that, treatment with empagliflozin increased energy expenditure (i.e: empagliflozin-treated mice exhibited higher oxygen consumption and exhaled more CO2 than the HFD-fed mice did, suggesting that treatment with empagliflozin enhances use of carbohydrates as well as fats under HFD conditions) suggested an increase in adaptive thermogenesis. Empagliflozin increased adipose tissue browning (the mRNA expression of brown fat-selective genes was also significantly increased in the empagliflozin treated group). empagliflozin alleviated obesity-associated insulin resistance (as detected by GTT, ITT and serum insulin level) and inflammation (as detected by significant decrease in inflammatory and proinflammatory markers) in the WAT and liver of obese mice.

Empagliflozin consistently reduced liver TG content and plasma levels of TG and TC in HFD-fed mice, and these findings were associated with the suppression of lipogenic gene expression and upregulation of mitochondrial fatty acid β-oxidation genes. Moreover, empagliflozin tended to upregulate liver fibroblast growth factor-21 (FGF21) protein level and markedly increased levels of plasma ketone bodies and glycerol, suggesting that lipolysis is enhanced in empagliflozin-treated mice. In addition, empagliflozin significantly decreased levels of plasma ALT and AST (empagliflozin alleviated HFD-induced liver damage) **(Xu et al., 2019).**

Theese results also are in agreement with **Adingupu et al., 2019,** whom studied the effects of empagliflozin 1.5 mg/kg body weight/day on coronary microvascular function and cardiac contractility in prediabetic ob/ob$^{-/-}$ mice and showed that, empagliflozin treated animals experienced a significant reduction in serum levels of ALT, AST, total cholesterol, TG, fasting glucose, insulin NAS score and body weight There was a statistical difference in liver steatosis score between treated and untreated mice. Empagliflozin reduced glycemia, promoted caloric loss via glucose excretion and reduced body weight gain. SGLT2 inhibitors mediate a metabolic switch from glucose to lipid utilization **(Ferrannini et al., 2014).**

This study also is in line with **Mizuno et al., 2018,** whom studied the effects of empagliflozin 10 mg/kg per day on the size and number of mitochondria after myocardial infarction in diabetic hearts and found that, empagliflozin significantly decreased serum glucose, total cholesterol, and triglyceride. The treatment with empagliflozin increased lipolysis.

But **Mizuno et al., 2018,** showed that, empagliflozin increased lipid droplets and TG level in the myocardium. **Mizuno et al., 2018,** explained that by, since the rate of fatty acid uptake into cardiomyocytes is dependent on the blood concentration of FFA, it is possible that the lipid accumulation induced by empagliflozin resulted from elevation of the circulating FFA level, possibly caused by the 24- h fasting induced lipolysis in the setting of their experiments. They speculated that reduced transport of FFA to the mitochondria might also be involved in the accumulation of lipid droplets in cardiomyocytes.

These results are in accordance with **Sawada et al.,** , as they studied the effects of empagliflozin on plasma triglyceride in diabetic patients with coronary artery disease and found that; after the 6 month

treatment, body weight and body fat percentage decreased significantly, plasma triglyceride, HbA1C level and fasting and postprandial plasma glucose levels were significantly decreased by treatment. Postprandial insulin secretion was also significantly suppressed and insulin resistance index was significantly decreased. Furthermore, fasting and postprandial triglyceride levels decreased significantly.

Sawada et al., 2019, noted that 6 months of treatment with empagliflozin improved glycemic control, obesity, insulin resistance, hypertriglyceridemia. In addition, it ameliorated inflammation. Improvement in both fasting and postprandial hypertriglyceridemia was the strongest independent factor predicting improvement of endothelial dysfunction in diabetic patients with CAD, treated with empaglifolozin. Empagliflozin indirectly improve insulin sensitivity by reducing glucose toxicity **(Abdul-Ghani et al., 2011).** Furthermore, weight reduction results from calorie loss because of glucosuria and lipid breakdown **(Cai et al., 2017).**

The results of the current study showed that, administration of silymarin at a dose of 100 mg/ kg ameliorated HFD induced NAFLD by significantly decreasing insulin resistance (decreased serum insulin level), decreasing markers of acute liver injury (decreased serum ALT and AST) and decreasing serum and hepatic lipid content (total serum cholesterol, serumTG and hepatic TG) and decreasing NAS score compared to HFD group (group II). Silymarin produced a non-significant change in random blood glucose and body weight as compared to HFD group.

This study is in accordance with that obtained by **Mahmoud et al., 2018,** whom studied the effects of silymarin 100 mg/kg/ day by means of oral gavage on high-fat-induced fatty liver in rats and found that, silymarin significantly decreased ALT, AST, total serum cholesterol, serum TG, hepatic TG, serum insulin level and NAS score as compared to HFD.

Multiple biochemical, metabolic and signal transduction pathways contribute to insulin resistance. It was found that protein glycation may play a role in insulin resistance by a variety of mecha-nisms, including generation of tumor necrosis factor-alpha, direct modification of the insulin molecule leading to its impaired action, generation of oxidative stress and impairment of mitochondrial function **(Song et al., 2012).** So, the beneficial effect of silymarin is mostly due improvement of insulin resistance (**Mahmoud et al., 2018).**

These results accordnates with a previous report, in which silymarin treatment was associated with a reduction of insulin resistance and a significant decrease in fasting insulin levels, suggesting an improvement of the activity of endogenous and exogenous insulin **(Cacciapuoti et al., 2013),** also **Federico et al., 2006,** showed that a new silybin vitamin E complex improves insulin resistance and liver damage in patients with NAFLD.

The results of the current work are in line eith **Kheiripour et al., 2019**. They studied the hepatoprotective effects of silymarin on liver injury and found that, silymarin resulted in a significant reduction of serum AST, ALT, random blood glucose, serum insulin and body weight.

Silymarin has many beneficial capabilities such as antioxidant, anti-inflammatory, immune-modulatory, and liver-regenerating capacities **(Surai et al., 2015; Kheiripour et al., 2019).**

Silymarin inhibits liver injury by maintaining the integrity of the plasma membrane, inhibits the secretion of liver enzymes in blood, and suppresses apoptosis in hepatocytes **(Vargas-Mendoza et al., 2014; Kheiripour et al., 2019)**

But this study doesn't agrees with that obtained by **Ni and Wang, 2016,** whom studied the effects of Silymarin on hepatic steatosis through regulation of lipid metabolism and oxidative stress in a mouse model of non-alcoholic fatty liver disease (NAFLD) and found that, silymarin produced a slight non-significant decrease in ALT, AST, body weight. But this study was conducted on mice that were fed with HFD for 20 weeks and treated with silymarin 30 mg/ kg only for 4 weeks only. But they showed that silymarin significantly decreased serum total cholesterol, serum TG, hepatic TG and histopathological changes induced by HFD.

Gobalakrishnan et al., 2016, studied the effects of silybin on lipid profile in hypercholesterolaemic rats and found that treament with Silybin significantly decreased serum total cholesterol serum TG and hepatic TG.

In silybin treated rats the fecal excretion of bile acids was increased. Enhanced excretion of faecal bile acids by Silybin depletes the pool of bile acids, and hepatic bile acid synthesis increases. As a result, hepatic cholesterol declines, stimulating the production of hepatic LDL receptors leading to enhanced clearance of plasma LDL, IDL and indirectly that of VLDL. This could be a possible mechanism of hypolipidemic effect of Silybin **(Gobalakrishnan et al., 2016).**

In the present study, empagliflozin significantly ameliorated antioxidant and anti-inflammatory effects induced by NAFLD (as it decreased hepatic levels of MDA, TGF-β and significantly increased hepatic GSH level).

The results of this work are in accordance with that obtained by **Abdelhamid et al., 2020,** whom studied the effects of Empagliflozin on ethanol-

induced liver injury and found that; empagliflozin significantly decreased hepatic level of MDA and increased hepatic GSH level. empagliflozin significantly decreased serum levels of alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase as markers of liver injury. Moreover, empagliflozin inhibited the release of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6 as markers of inflammation,via the downregulation of NF-κB. These changes were associated with an improvement in histopathological deterioration. In conclusion **Abdelhamid et al., 2020** showed that, empagliflozin modulated alcohol-induced liver damage by suppressing oxidative stress and inflammatory responses.

These results suggest that empagliflozin exhibits remarkable free radical scavenging activities and can reverse the reduction in the activities of antioxidant enzymes. In accordance with the present results, previous studies have demonstrated that empagliflozin could act as an antioxidant by decreasing oxidative stress and enhancing antioxidative enzymes, independently from its known glucose-lowering activity **(Kabel et al., 2020 and Lahnwong et al., 2018).**

Empagliflozin showed many benefits potentially mediated through its ability to reduce inflammation, oxidative stress, apoptosis, mitochondrial dysfunction and ionic dyshomeostasis **(Amin et al., 2020 and Yaribeygi et al., 2019).**

The current study also coincides with **Li et al., 2019,** whom studied the effect of empagliflozin on myocardial oxidative stress and fibrosis in diabetic mice heart and found empagliflozin to decrease hepatic level of MDA and increase hepatic GSH level. They confirmed that, empagliflozin could effectively control blood glucose and reduce insulin release. In addition to its role in good glycemic control, empagliflozin also significantly lowers blood cholesterol and TG levels in diabetic mice.

Empagliflozin inhibited the classical TGF-β/Smad pathway and decreased cardiac oxidative stress **(Li et al., 2019).**

Regarding the antioxidant and anti-inflammatory effects of silymarin, it significantly decreased hepatic level of MDA, TGF-β and increased hepatic GSH level.

In concidance with our results, **Mahmoud et al., 2018,** found that, administration of silymarin resulted in a significant reduction of hepatic MDA, TGF-β and increased hepatic GSH level significantly. Silymarin has demonstrated antioxidant properties, through stimulation of polymerase and RNA transcription, by protecting the cell membrane from radical-induced damage and blocking the uptake of toxins **(Wellington et al., 2001).**

This study is in line with **Yao et al., 2013**. They studied the effect and the probable mechanisms of silibinin in regulating insulin resistance in the liver of rats with non-alcoholic fatty liver and found that, compared with the model group; silybin treatment significantly decreased hepatic malondialdehyde (MDA) and increased hepatic GSH level, which coincides with the recent study. Also silibinin significantly decreased visceral fat, serum total cholesterol, TG, ALT, AST and IR.

This study also is in accordance with **Mahli et al., 2015,** whom studied the hepatoprotective effect of oral application of a silymarin extract in carbon tetrachloride-induced hepatotoxicity in rats and found that, silymarin resulted in a significant reduction of hepatic TGF-β compared to NAFLD non-treated group. They found that the silymarin extract had direct inhibitory effects on pro-inflammatory and pro-fibrogenic gene expression in HSCs in vitro. This indicates that direct effects on HSC also contribute to the in vivo hepatoprotective effects of silymarin, and further promote its potential as anti-fibrogenic agent also in chronic liver disease.

Histopathological study of empagliflozin treated group showed less frequent inflammatory foci, moderate steatosis and mild perisinasoidal fibrosis compared to HFD group.

This histopathology coincides with **Jojima et al., 2016** and **Xu et al 2017**whom found that; empagliflozin significantly improved fatty degeneration, inflammatory cell infiltration and hepatocellular ballooning and TG content..

Histopathological examination of silymarin treated group showed marked steatosis. hydrobic changes, inflammatory foci with marked perisinasoidal fibrosis.

This study agrees with **Mahmoud et al., 2018,** whom found that, administration of silymarin resulted in a significant decrease of pathological changes induced by high fat diet feeding and improvement of histopathological scores in liver samples showing mild to moderate steatosis and less inflammation and ballooning.

Silymarin has an anti-inflammatory effect by acting through different mechanisms as its antioxidant action, membrane stabilizing effect and inhibition of production or release of inflammatory mediators **(Mahmoud et al., 2018).**

Administration of silibinin for 6 weeks significantly improved fatty degeneration and inflammation (manifesting as less cytological ballooning, relatively regularly arranged cell cords and decreased lobular inflammation). In addition silymarin reduced fat accumulation in hepatocytes **(Yao et al., 2013).**

This work also accords with that obtained by **Ni and Wang, 2016,** whom found that, silymarin significantly reversed HFD induced histopathologic changes that induced by HFD. silymarin could attenuate hepatic steatosis in a mouse model of NAFLD through regulation of lipid metabolism and oxidative stress, and benefit to the circulation system

As compared to empagliflozin, in the present study, silymarin decreased ALT, AST, and hepatic MDA more significantly, but vildagliptin significantly decreased total serum cholesterol, serum TG, random blood glucose and hepatic TG more significantly compared to silymarin. Silymarin increased GSH and decreased TGF-β more significantly as compared to empagliflozin. Empagliflozin dcreased body weight significantly while silymarin produced non-significant decrease in the body weight. Empagliflozin reduced serum insulin level and NASH score more significantly than silymarin.

The beneficial effects of empapagliflozin could be explained by that, empagliflozin is a potent oral anti-diabetic agent that inhibits sodium-glucose cotransporter 2 (SGLT-2). By inhibiting this transporter, SGLT-2 inhibitors promote urinary glucose excretion, which, in turn, decreases blood levels of glucose and improves insulin resistance in patients with type 2 diabetes leading to down-regulation of carbohydrate-responsive element-binding protein (ChREBP), a transcription factor responsible for activating the machinery for fatty acid synthesis. Improvement in insulin resistance (hyperinsulinemia) results in down-regulation of SREBP-1c and the blockage of hepatic lipogenesis **(Kuchay et al., 2018).**

Empagliflozin markedly decreased obesity induced inflammation in the liver of diet induced obese (DIO) mice **(Xu et al., 2017).** These findings suggest that SGLT2 inhibitors, particularly empagliflozin, improve insulin resistance partially by attenuating chronic inflammation in obese and diabetic subjects **(Xu and Ota, 2018).**

Consistent with other SGLT2 inhibitors, administering empagliflozin to HFD-induced obese mice mitigates weight gain and fatty liver. The underlying mechanism for the weight reduction depends partially on increased energy expenditure and enhanced fatty acid oxidation. Empagliflozin increases oxygen consumption and tends to elevate carbon dioxide exhalation, leading to increased sugar and fat utilization **(Xu et al., 2017)**.

Empagliflozin increases the plasma LDL-C level concomitant with higher FFAs and total ketone body levels, which suggests that inhibiting SGLT2 induces ketogenesis and a metabolic switch toward lipid oxidation to counterbalance the carbohydrate restriction **(Briand et al., 2016).**

Administering empagliflozin increases fatty acid oxidation by altering the expression of adiponectin and leptin in epididymal WAT. The adipose tissue-specific adipokines; leptin and adiponectin are involved in the regulation of food intake and energy homeostasis **(Adya et al., 2015).**

Plasma leptin stimulates fatty acid esterification to triglycerides and causes an even greater increase in hydrolysis so that there is a net efflux of fatty acids from the cells. By contrast, adiponectin exerts its insulin-sensitizing effects by increasing β-oxidation of fatty acids and reducing serum triglyceride and FFA levels, thus indirectly improving insulin sensitivity. Furthermore, leptin and adiponectin interact with AMPK, which regulates fatty acid and energy metabolism. Administering empagliflozin increases adiponectin mRNA expression and down-regulates leptin expression in epididymal WAT and contributes to fat lipolysis and energy expenditure. Taken together, these findings indicate that empagliflozin improves abnormal lipid metabolism and obesity by enhancing fat and sugar utilization and increasing fatty acid oxidation **(Xu and Ota, 2018).**

Zhao et al., 2016, showed elevation of adiponectin down-regulates the renal SGLT2 by activating PPARδ, which in turn reduces reabsorption of sodium and glucose **(Zhao et al., 2016).** On the other hand, SGLT2 inhibitors increase the expression of adiponectin in diabetic subjects and obese model **(Xu et al., 2017; Tanizawa et al., 2013)**.Empagliflozin promotes adiponectin expression in WAT **(Xu et al., 2017)**.

Treatment with SGLT2i(s) results in decreases in both glucose and insulin levels (especially in those with T2DM) which lead to a large decrease in hepatic de novo lipid synthesis **(Daniele et al., 2016).**

Glucagon-secreting alpha cells of pancreatic islets express SGLT2, and inhibition of the SGLT2 co-transporter (which presumably reduces intracellular glucose concentration in these cells an important signal for glucagon release) results in increased secretion and blood levels of glucagon **(Daniele et al., 2016; Wang et al., 2018).**The high glucagon levels (and elevated glucagon/insulin ratio) lead to stimulation of β-oxidation (and hepatic ketone production and elevated blood ketone levels) and cause a shift from carbohydrate to fatty acid metabolism and help reduce liver triglyceride content ((**Daniele et al., 2016).**

In keeping with the importance of glucagon in the pathogenesis of NAFLD, a recent study showed that while a reduction in glucagon receptor expression (using antisense technology) in patients with T2DM improved glycemic control, it also increased their ALT levels and significantly increased hepatic fat content **(Morgan et al., 2019).**

As compared to vildagliptin treated group, empagliflozin treated group showed non-significant changes in serum ALT, AST, significant decrease in total serum cholesterol, serum TG, non-significant changes in random blood glucose, hepatic MDA, GSH, significant decrease in hepatic TG, non-significant changes in hepatic TGF-β, body weight, significant decrease in serum insulin level and non-significant decrease in NASH score.

Silybin could be an insulin sensitizer; as it is able to reduce intrahepatic fat accumulation, lobular inflammation, ballooning and serum fat, as well as to improve homeostasis model assessment-IR index (HOMA-IR) and insulin tolerance test (ITT). Moreover, silybin has an important role in reducing visceral fat accumulation, in inducing lipolysis through the transcription of the adipose triglyceride lipase (ATGL) gene and inhibiting gluconeogenesis for silencing of some genes involved in the fat metabolic pathway **(Yao et al., 2013).**

Silybin has low bioavilability if administered per orally. It reduces the absorption of fat contained in HFD mediated by the formation of non-absorbable complexes, rather than depending on its real role in interrupting the pathogenetic mechanisms that are responsible for NAFLD **(Christodoulou et al., 2015).**

The treatment with increasing doses of silybin (16, 40 and 100 µg/mL), in vitro, is able to generate an increase in glucose captation, induced by insulin in a model of palmitate-induced insulin-resistance on myoblast C2C12 cells, in which the role of silybin is crucial **(Li et al., 2015).**

Conclusion:

Empagliflozin and silymarin had ameliorated HFD induced biochemical, inflammatory, oxidative stress and histopathological changes, but empagliflozin was more effective and more significant. So, more research is needed to investigate the effectiveness of these drugs in NAFLD.

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