

# IRISIN: A POTENTIAL PROMISING AGENT AGAINST NON-ALCOHOLIC FATTY LIVER DISEASE IN TYPE 2 DIABETIC RATS

By

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## ABSTRACT

**Background:** Non-alcoholic fatty liver disease (NAFLD) is strongly related to obesity, insulin resistance and type 2 diabetes. Irisin is an exercise-induced myokine which improves glucose profile and insulin resistance.

**Objective:** To evaluate the potential effects of irisin on liver function in type 2 diabetic rats with NAFLD and elucidate the underlying mechanisms.

**Material and methods:** Thirty adult male albino rats were divided into 3 equal groups: control rats, type 2 diabetic with NAFLD rats, and type II diabetic rats with NAFLD received irisin (0.5 µg/g body weight once daily for 8 weeks). Diabetes was induced by high fat diet for 12 weeks followed by injection of small dose of streptozotocin (35 mg/kg body weight). Eight weeks after establishment of diabetes, body mass index and abdominal circumference were estimated. Serum was analysed for glucolipid metabolic parameters, liver enzymes, C-reactive protein, tumour necrosis factor-α. Liver homogenate was assayed for malondialdehyde, superoxide dismutase, and glutathione s-transferase activities. Liver sections were stained with Hematoxylin & eosin.

**Results:** Irisin treatment significantly improved glucolipid metabolic parameters, liver enzymes, antioxidant enzymes accompanied by significant decrease in inflammatory mediators and malondialdehyde levels, with improvement of the histopathological findings.

**Conclusion:** Irisin may be used as a promising agent against NAFLD in type 2 diabetic rats possibly through controlling glucose and lipid metabolism and via its beneficial anti-inflammatory and anti-oxidant effects as well.

**Keywords:** Irisin, Type 2 Diabetes, Fatty Liver, High- Fat Diet, Rats.

## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease and is characterized by diffused steatosis and hepatocyte lipid accumulation without substantial alcohol intake. NAFLD may progress to non-alcoholic steatohepatitis (NASH), leading to liver fibrosis and hence cirrhosis (Sanyal et al., 2011 and El-Sherbiny et al., 2018). Further, it is associated with

hyperlipidemia, obesity and type II diabetes (Higuera de la Tijera and Servín Caamaño, 2015). Moreover, it is associated with a high mortality rate due to an increased risk of cardiovascular disease and hepatoma (Hassan et al., 2014). The complexity of NAFLD pathogenesis is multifactorial and explained by the “multiple hit theory”, where many factors, such as genetic predisposition, insulin resistance, altered

adipokine production, endoplasmic reticulum stress and mitochondrial dysfunction, are involved. These factors could also explain the various disease patterns among NAFLD patients (*Buzzetti et al., 2016*).

In the early stages of NAFLD, the disease can be managed by lifestyle changes in the form of weight reduction and exercise (*Arias-Loste et al., 2014*). However, the advanced conditions are treated by adding insulin sensitizers, lipid lowering agents and antioxidants as well (*Fruci et al., 2013; Del Ben et al., 2014* and *Leite et al., 2014*). Unfortunately, these drugs have potential long-term side effects. Therefore, there is a considerable demand to develop effective and safe therapies for the management of NAFLD (*Issa et al., 2018*).

Irisin, is a 112 amino acid myokine as a cleaved form of fibronectin type III domain-containing protein 5 (FNDC5) (*Boström et al., 2012*), this myokine secreted chiefly in skeletal and cardiac muscles. However, little is detected in adipose tissue and liver (*Aydin et al., 2014*). Physiologically, irisin release from skeletal muscles is increased following exercise and ATP depletion (*Löffler et al., 2015; Palacios-González et al., 2015* and *Panati et al., 2016*). Hence, it can induce browning of white adipose tissue, increasing heat production and energy expenditure (*Virtanen, 2014*).

Irisin was found to stimulate glucose uptake by skeletal muscles, improving liver glucose and lipid metabolism and leading to the relief of hyperglycemia, insulin resistance and hyperlipidemia in obesity-related conditions (*Chen et al., 2016* and *So & Leung, 2016*).

This study aimed to evaluate the potential effects of irisin on liver function in type 2 diabetic rats with NAFLD and elucidate the underlying mechanisms.

## MATERIALS AND METHODS

- **Animals:** Thirty healthy adult male Wistar albino rats weighing 180-210 g were obtained from the Faculty of Veterinary Medicine, Zagazig University. The animals were kept under hygienic conditions in steel wire cages (40cm 28cm 18cm- 5rats/ cage) in the animal facility of the Physiology Department, Faculty of Medicine, Zagazig University. Animals had free access to water and were maintained at room temperature on a 12 - hour light/dark cycle. The rats were acclimatized to the animal facility conditions for one week before the experiments. The experimental protocols were approved by the Zagazig University Institutional Animal Care Unit Committee (ZU-IACUC; Sharqia, Egypt).

This study was performed from August, 2018 to February, 2019. After acclimation for one week, the rats were randomly assigned to three equal groups. **In Group I** (normal controls), the rats were fed a standard normal chow diet consisting of 25.8% protein, 62.8% carbohydrates and 11.4% fat (as a percentage of total kcal) for 12 weeks (*Ahrén and Scheurink, 1998*). **In Group II** (type II diabetic rats with NAFLD), a type 2 diabetes model was established by feeding animals a high-fat diet (HFD) consisting of 58.8 % fat, 26% carbohydrates and 15.2% protein (as a percentage of total kcal) (The diets were obtained from The Agriculture Faculty,

Zagazig University. After twelve weeks of dietary manipulation, all rats were fasted for 12 hours with free access to water. Then, the rats received an intraperitoneal injection of low dose streptozotocin (STZ) (35 mg/kg body weight) (Sigma Chemical, St. Louis, MO, Sigma-Aldrich, U.S.A). However, the control rats fed a standard normal chow diet were given an equivalent volume of saline. Blood glucose was checked seven days after STZ injection. Animals with blood glucose levels  $\geq 250$  mg /dl were chosen for the study (Hu et al., 2013). This group was maintained for eight weeks after induction of diabetes with NAFLD. **In Group III** (type II diabetic rats with NAFLD received irisin), diabetic rats received intraperitoneal injections of irisin (Phoenix Pharmaceuticals, Inc. Burlingame, USA) at a dose of 0.5 ?g/g body weight once daily for 8 weeks (Zhang et al., 2014a and Xin et al., 2015). The control and untreated diabetic groups (I and II) received intraperitoneal injections of phosphate buffered solution (PBS) at the same dose and duration as irisin (Xin et al., 2015).

#### Measurement of anthropometric parameters:

- Body mass index (BMI) (g/cm<sup>2</sup>) was calculated by dividing body weight (gm) by length<sup>2</sup> (cm<sup>2</sup>). The obesity cut-off value was a BMI above 0.68 g/cm<sup>2</sup> (Novelli et al., 2007).
- Abdominal circumference (AC), rats were placed in the ventral position, and the abdominal circumference was assessed in the largest zone of the rat's abdomen using a plastic non-extensible measuring tape (Gerbaix et al., 2010).

**Blood sampling and biochemical analysis:** Blood samples were obtained from the retro-orbital venous plexus at the end of the experimental period between 9-11 A.M. after overnight fasting (Yang et al., 2006 and Lemos et al., 2011). The blood was collected in plastic centrifuge tubes and allowed to clot. Serum was separated by centrifugation at 3000 rpm for 15 minutes. The supernatant serum was pipetted off and stored frozen at -20 °C (Nishizawa et al., 2002) until assayed for assessment of the following:

#### 1. Serum glucose, insulin levels and insulin resistance

- Serum glucose levels (mg/dl) were measured as described by Tietz (1995), and serum insulin levels (?IU/ml) were measured by enzyme linked immunosorbent assay (ELISA) according to Reaven (1991). Kits for serum glucose and insulin levels were purchased from Biosource Europe S.A. Belgium.
- Homeostasis model assessment-insulin (HOMA-IR) was calculated using the HOMA-IR index according to the equation used by Bonora et al. (2000):  $HOMA-IR = \text{fasting serum glucose (mg/dL)} \times \text{fasting serum insulin (?IU/mL)} / 405$ .

#### 2. Lipid profile

- Serum total cholesterol (TC) and triglycerides (TG) levels were measured by an enzymatic colorimetric method using specific cholesterol and triglycerides kits (Spinreact Spain), and analysed by a spectrophotometer as described in previous studies (Fossati and Prencipe, 1982 and Tietz, 1995). HDL-c levels were measured as described by Nauck et al. (1997).

Serum LDL-c levels were calculated by using the Friedewald formula (*Friedewald et al., 1972*) as follows:  $LDL-c = TC - HDL - TG/5$ .

### 3. Liver enzyme assays

- Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured via a Rat ALT ELISA kit (Kamiya Biomedical Company, KT-6104, Gateway Drive, Seattle) and a Rat AST ELISA kit (Sunred Biological Technology, China), respectively, according to the method of *Vassault (1983)*.

### 4. Serum albumin

- Serum albumin was estimated by using bromocresol green according to the method described by *Stoskopf (1993)*.

### 5. Serum irisin level

- Serum irisin was measured as described by *Boström et al. (2012)* using an irisin ELISA rat kit (Catalogue # K4761-100, BioVision, Milpitas Blvd., Milpitas, CA 95035 USA).

### 6. Inflammatory markers

- TNF- $\alpha$  concentration was measured by ELISA as described by *Fernando et al. (1998)* using commercial kits purchased from ALPCO (45-TNFRT-E01.1). Absorbance was measured at 450 nm.
- C-reactive protein (CRP) was estimated by kits (Monobind Inc., Lake Forest, Ca 92630, USA) as described by *Kimberly et al. (2003)*.

### Assessment of oxidative stress markers in liver homogenate:

Liver tissues were processed to obtain 10% (w/v) homogenate in ice-cold 20 mM

tris (hydroxymethyl) amino-methane buffer (pH 7.4). Homogenates were centrifuged at  $3000 \times g$  for 30 minutes at 4 °C. The supernatants were collected and assayed as follows:

- Hepatic malondialdehyde (MDA), a lipid peroxidation indicator, was estimated using a Bio diagnostic kit according to *Varshney and Kale (1990)*.
- Hepatic superoxide dismutase (SOD) was determined using phenazine methosulfate (PMS) depending on the SOD ability to inhibit the PMS-mediated reaction of nitro-blue tetrazolium as described by *Misra and Fridovich (1972)*.
- Glutathione S-transferase (GST) activity was assayed using 1-chloro-2, 4-dinitrochlorobenzene as a substrate according to (*Rajurkar et al., 2003*). A GST unit is defined as 1 mol of CDNB-GSH conjugate formed/min/mg protein.

### Liver extraction and histopathological examination:

After blood sample collection, the rats were sacrificed by cervical dislocation under mild isoflurane anesthesia, and then laparotomy was conducted. The livers were quickly removed, cleaned and processed for histopathological examinations. The excised livers were fixed in 10% buffered formalin solution for 48-60 hours. Then, tissue samples were processed through an ethyl alcohol and xylene series and embedded in paraffin blocks. Liver specimens were sectioned (5  $\mu$ m thick) and then stained with hematoxylin and eosin (*Altunkaynak, 2005*). A pathologist evaluated and scored the stained samples in a blind fashion

using a light microscope with camera attachment. The histological scoring of NAFLD followed the NAFLD Activity Score (NAS) proposed by The Pathological Committee of the NASH Clinical Research Network (Kleiner *et al.*, 2005). The scores are as follows: steatosis (0 = <5%, 1 = 5%– 33%, 2 = 34%– 66%, 3 = >66%), lobular inflammation (0 = no foci, 1 = <2 foci per 200 × field, 2 = 2–4 foci per 200 × field, 3 = >4 foci per 200 × field), and ballooning (0 = none, 1 = rare or few, 2 = many or prominent). Fibrosis staging was recorded as the following criteria: 0 = none, 1 = perisinusoidal or periportal fibrosis, 2 = perisinusoidal and portal/periportal fibrosis, 3 = bridging fibrosis and 4 = cirrhosis. A NAS score  $\geq$  5 was defined as NASH;  $2 < \text{NAS} < 5$  was defined as borderline NASH; and  $\text{NAS} \leq 2$  was simple steatosis (Zhao *et al.*, 2004). Cirrhosis was defined as grade 4 fibrosis plus other features of the NAS score.

#### **Immunohistochemical staining with anti-caspase-3 antibody:**

Liver slides were stained with rabbit anti-Caspase-3 antibody (cat# ab4051, Abcam, USA) to identify apoptotic areas.

The deparaffinized sections were bathed in phosphate-buffered saline, and endogenous peroxidase activity was obstructed by incubation with 3% hydrogen peroxide for 10 minutes at room temperature. Then, sections were incubated with a primary antibody overnight to detect the presence of apoptosis markers. Liver sections were counterstained with hematoxylin (El-Sherbiny *et al.*, 2018).

According to the intensity of the staining, caspase 3 expression was scored into 3 grades: 1+ weak or negative, 2+ moderate, and 3+ strong (Dozic *et al.*, 2016).

#### **Statistical analysis:**

Statistical analysis was performed by using SPSS 19 (SPSS Inc. Chicago, IL, USA). ANOVA with Post hoc (LSD) was used to compare means among the groups. The correlations between parameters were analyzed using Pearson's correlation. P value < 0.05 was considered indicative of statistical significance.

## **RESULTS**

**In group II** (Type II diabetic rats with NAFLD), there was a significant increase in final body weight, liver weight, liver/body weight ratio, AC, final BMI, serum glucose, insulin, HOMA-IR, cholesterol, triglycerides, LDL, ALT, AST, TNF- $\alpha$ , CRP and hepatic MDA (**P < 0.001**) in comparison to **group I** (control). However, there was a significant decrease in serum irisin, HDL, albumin, hepatic SOD and hepatic GST (**P < 0.001**) in the same group [Tables 1, 2, 3].

**In group III** (Type II diabetic rats with NAFLD received irisin), irisin administration resulted in a significant decrease in final body weight, liver weight, liver/body weight ratio (**P < 0.01**), AC (**P < 0.05**), final BMI, serum glucose, insulin, HOMA-IR (**P < 0.001**), cholesterol, triglycerides, LDL (**P < 0.01**), ALT, AST (**P < 0.001**), TNF- $\alpha$ , CRP (**P < 0.01**) and hepatic MDA (**P < 0.001**), but significantly increased serum HDL (**P < 0.01**), irisin, albumin, hepatic SOD, and hepatic GST (**P < 0.001**) in comparison to

**group II** (Type II diabetic rats with NAFLD) [Tables 1, 2, 3].

Furthermore, There was significant negative correlation between irisin levels and each of final BMI, AC, serum glucose, insulin, HOMA-IR, cholesterol, triglycerides, LDL, HDL, albumin, ALT,

AST, TNF- $\alpha$ , CRP and hepatic MDA in all studied groups. However, was significant positive correlation between irisin levels and each of hepatic SOD and hepatic GST in all studied groups [Tables 1, 2, 3].

**Table (1): Anthropometric parameters in all studied groups**

Parameters		Groups	Group I (control)	Group II (Type II diabetic rats with NAFLD)	Group III (Type II diabetic rats with NAFLD received irisin)
Final body weight (gm)	$\bar{X} \pm SD$		196.62±13.2	281.50±13.9 †***	247.62±21.3 †***§**
Liver weight (gm)	$\bar{X} \pm SD$		8.32±0.6	13.80±0.8 †***	12.35±0.7 †***§**
Liver/ body weight ratio (100%)	$\bar{X} \pm SD$		4.25±0.1	5.01±0.1 †***	4.8±0.1 †***§**
Final BMI (gm/cm <sup>2</sup> )	$\pm SD \bar{X}$ (r)		0.58±0.04 (- 0.819 <sup>*</sup> )	0.88±0.07 †*** (- 0.832 <sup>*</sup> )	0.68±0.04 †***§** (- 0.771 <sup>*</sup> )
AC (cm)	$\pm SD \bar{X}$ (r)		17.50±1.9 (-0.726 <sup>*</sup> )	22.00±2.00 †*** (-0.835 <sup>**</sup> )	19.79±2.1 †**§* (-0.741 <sup>*</sup> )

†= vs group I; §= vs group II; r =correlation coefficient versus irisin level;\*= significant (P<0.05); \*\*= significant (P<0.01); \*\*\*= significant (P<0.001).

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**Table (2): Serum biochemical parameters in all studied groups**

Parameters \ Groups		Group I (control)	Group II (Type II diabetic rats with NAFLD)	Group III (Type II diabetic rats with NAFLD received irisin)
Glucose (mg/dl)	$\pm$ SD $\bar{X}$	82.37 $\pm$ 7.4	270 $\pm$ 20 ††††	187 $\pm$ 20 ††††§§§§
	(r)	(- 0.829*)	(- 0.843**)	(- 0.857**)
Insulin (?IU/ml)	$\pm$ SD $\bar{X}$	17.3 $\pm$ 1.2	28.82 $\pm$ 3.9 ††††	22.82 $\pm$ 1.9 ††††§§§§
	(r)	(- 0.765*)	(- 0.837*)	(- 0.827*)
HOMA-IR	$\pm$ SD $\bar{X}$	3.88 $\pm$ 0.4	19.4 $\pm$ 2.1 ††††	10.5 $\pm$ 0.5 ††††§§§§
	(r)	(- 0.908**)	(- 0.882**)	(- 0.862**)
Cholesterol (mg/dl)	$\pm$ SD $\bar{X}$	90.37 $\pm$ 4.4	141.75 $\pm$ 3.9 ††††	127.12 $\pm$ 12.4 ††††§§§§
	(r)	(- 0.830*)	(- 0.743*)	(-0.728*)
Triglycerides (mg/dl)	$\pm$ SD $\bar{X}$	52.25 $\pm$ 4.8	100.75 $\pm$ 12.4 ††††	88.37 $\pm$ 6.4 ††††§§§§
	(r)	(- 0.831*)	(- 0.849**)	(- 0.845**)
HDL (mg/dl)	$\pm$ SD $\bar{X}$	40.62 $\pm$ 1.5	34.25 $\pm$ 1.5 ††††	37.37 $\pm$ 1.7 ††††§§§§
	(r)	(- 0.930**)	(- 0.898**)	(- 0.751*)
LDL (mg/dl)	$\pm$ SD $\bar{X}$	30.64 $\pm$ 4.6	75.04 $\pm$ 7.3 ††††	63.55 $\pm$ 6.2 ††††§§§§
	(r)	(- 0.885**)	(- 0.902**)	(- 0.762*)
Irisin (ng/ml)	$\pm$ SD $\bar{X}$	7.50 $\pm$ 1.2	4.55 $\pm$ 0.3 ††††	7.82 $\pm$ 0.8 ††††
Albumin (g/dl)	$\pm$ SD $\bar{X}$	3.92 $\pm$ 0.26	2.99 $\pm$ 0.23 ††††	3.53 $\pm$ 0.12 ††††§§§§
	(r)	(-0.844**)	(- 0.768*)	(- 0.742*)
ALT (U/L)	$\pm$ SD $\bar{X}$	39.97 $\pm$ 6	91.37 $\pm$ 10.7 ††††	55.51 $\pm$ 8.2 ††††§§§§
	(r)	(- 0.923**)	(- 0.780*)	(- 0.739*)
AST (U/L)	$\pm$ SD $\bar{X}$	78.87 $\pm$ 5.3	128.62 $\pm$ 11.9 ††††	93.87 $\pm$ 9.5 ††††§§§§
	(r)	(- 0.855**)	(- 0.938**)	(- 0.869**)
TNF- $\alpha$ (pg/ml)	$\pm$ SD $\bar{X}$	13.97 $\pm$ 1.3	45.55 $\pm$ 6.9 ††††	35.75 $\pm$ 5.2 ††††§§§§
	(r)	(- 0.870**)	(- 0.864**)	(- 0.865**)
CRP (mg/l)	$\pm$ SD $\bar{X}$	0.036 $\pm$ 0.008	0.105 $\pm$ 0.03 ††††	0.075 $\pm$ 0.006 ††††§§§§
	(r)	(- 0.899**)	(- 0.866**)	(- 0.813*)

†= vs group I; §= vs group II; r =correlation coefficient versus irisin level;\*= significant (P<0.05);  
 \*\*= significant (P<0.01); \*\*\*= significant (P<0.001).

Table (3): Oxidative stress markers in liver homogenate in all studied groups

Parameters		Group I (control)	Group II (Type II diabetic rats with NAFLD)	Group III (Type II diabetic rats with NAFLD received irisin)
MDA (nmol/g protein)	$\pm$ SD $\bar{X}$	1.59±0.2	3.49±0.7 <sup>‡***</sup>	2.17±0.2 <sup>‡***§***</sup>
	(r)	(-0.829 <sup>*</sup> )	(-0.833 <sup>*</sup> )	(-0.765 <sup>*</sup> )
SOD (U/mg protein)	$\pm$ SD $\bar{X}$	27.46±4.3	15.89±1.06 <sup>‡***</sup>	22.9±5.5 <sup>‡***§***</sup>
	(r)	(+0.763 <sup>*</sup> )	(+0.804 <sup>*</sup> )	(+0.732 <sup>*</sup> )
GST (U/mg protein)	$\pm$ SD $\bar{X}$	2.39±0.2	0.98±0.1 <sup>‡***</sup>	2.12±0.1 <sup>‡***§***</sup>
	(r)	(+ 0.750 <sup>*</sup> )	(+ 0.781 <sup>*</sup> )	(+ 0.742 <sup>*</sup> )

‡= vs group I; §= vs group II; r =correlation coefficient versus irisin level;\*= significant (P<0.05); \*\*= significant (P<0.01); \*\*\*= significant (P<0.001).

Hepatic histopathological examination revealed normal liver tissue with normal architecture, normal hepatocyte and normal central vein in **group I** (control) [Fig 1 A] and HFD induced non- alcoholic steatohepatitis with marked micro and macrosteatosis in hepatocytes with ballooning degeneration and lobular inflammatory infiltrate with congested central vein in **group II** (Type II diabetic rats with NAFLD) [Fig 1 B]. Furthermore, group III (Type II diabetic rats with NAFLD received irisin), showed significant improvement in the degree of the hepatic lesions with mild fatty change in form of small intracytoplasmic vacuoles and apoptotic bodies in comparison to **group II** [Fig 1 C].

There was a significant decrease in hepatic injury score in **group III** in comparison to **group II** (p<0.001) with a significant negative correlation between irisin level and hepatic injury score in **group II** and **group III** (p<0.01 and p<0.05 respectively) [Table 4].

Hepatic immunohistochemical images stained with anti- caspase 3 antibody showed significant increase in caspase 3 expression in **group II** in comparison to **group I** (p<0.001) which showed faint positive staining for caspase 3 . However, in group III there was significant decrease in caspase 3 expression when compared to group II (p<0.001) [Table 5, Fig 2 A, B, C].

Table (4): Histopathological scoring of liver injury induced by HFD

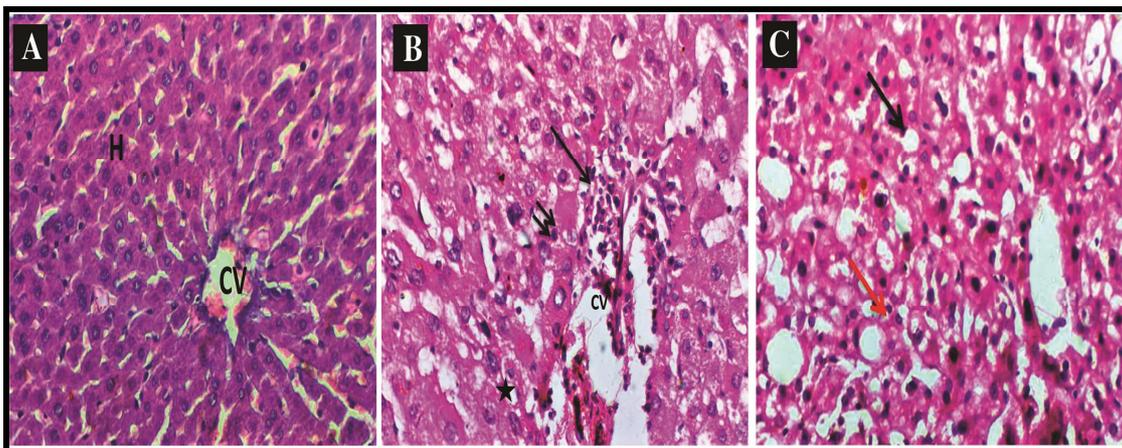
Parameters		Group I (control)	Group II (Type II diabetic rats with NAFLD)	Group III (Type II diabetic rats with NAFLD received irisin)
Hepatic injury score	$\bar{X} \pm$ SD	0	6.12±0.8 <sup>‡***</sup>	2.87±0.8 <sup>‡***§***</sup>
	r		- 0.885 <sup>**</sup>	- 0.714 <sup>*</sup>

‡= vs group I; §= vs group II; r =correlation coefficient versus irisin level;\*= significant (P<0.05); \*\*= significant (P<0.01); \*\*\*= significant (P<0.001).

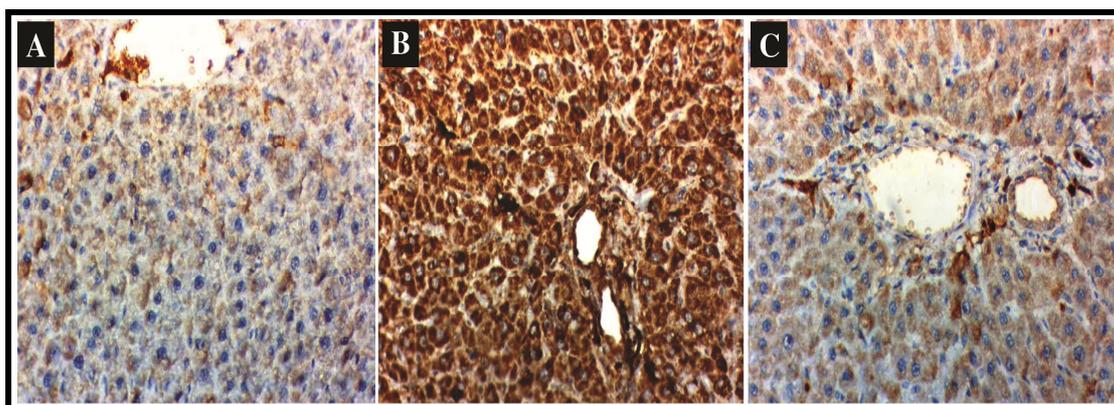
**Table (5): Immunohistochemical expression of caspase 3 in the three studied groups**

Parameters		Group I (control)	Group II (Type II diabetic rats with NAFLD)	Group III (Type II diabetic rats with NAFLD received irisin)
caspase 3 expression	$\bar{X} \pm SD$	0.75±0.5	2.87±0.3 <sup>‡***</sup>	1.62±0.7 <sup>‡***§***</sup>

‡= vs group I; §= vs group II; \*= significant (P<0.05); \*\*= significant (P<0.01); \*\*\*= significant (P<0.001).



**Figure (1): Liver tissue of rat showing: A) group I:** central vein (CV) surrounded with normal hepatocytes arranged in cords (H), **B) group II:** NASH with marked micro and macrosteatosis in hepatocytes (star) with ballooning degeneration (double arrow) and Lobular inflammatory infiltrate (arrow) with congested central vein (CV), **C) group III:** Cells show mild fatty change in form of small intracytoplasmic vacuoles (black arrow) and apoptotic bodies (red arrow) (H&E X400).



**Figure (2): Liver tissue of rat stained with caspase 3 showing: A) group I:** weak (1+) caspase 3 expression in normal hepatocytes, **B) group II:** strong (3+) caspase 3 expression, **C) group III:** moderate (2+) caspase 3 expression (IHC X400).

## DISCUSSION

In the current study, insulin resistance was induced via a HFD (*Jiang et al., 2013*), followed by a single STZ injection (35 mg/kg) to induce clear hyperglycemia. After 12 weeks of feeding, the rats were assessed to confirm NAFLD model establishment in the HFD fed- group (*Fan, 2007*). HFD/STZ-induced NAFLD in type 2 diabetic rats is characterized by typical hepatocellular injury associated with disturbed lipid, and glucose metabolism and subsequent liver steatosis. Extended HFD intake induced a significant increase in body weight, BMI, AC, fasting serum glucose and insulin levels, resulting in a higher HOMA IR compared with the control group. Furthermore, a HFD induced insulin resistance and disturbed glucose metabolism. Insulin resistance and dysregulation of fat metabolism are central players in the development of T2DM and NAFLD (*Xu et al., 2015*). Insulin resistance may initiate liver injury resulting in steatosis worsened by a HFD (*Bell et al., 2012; Liu et al., 2014; Xu et al., 2014* and *Ideta et al., 2015*). Therefore, improving insulin sensitivity ameliorates T2DM and NAFLD. Additionally, a long-lasting HFD worsened the lipid profile as follows: elevated serum TG, TC, and LDL-c levels and decreased HDL-c levels. Moreover, a long-term HFD resulted in liver dysfunction as indicated by increased serum liver enzymes (ALT and AST), liver weight, and decreased serum albumin. Moreover, in this study, histopathological examination of the livers of diabetic rats revealed liver injury in the form of NASH establishment indicated by fatty infiltration and foci of inflammatory

infiltration represented by spotty necrosis, and ballooning degeneration. Indeed, the histopathological findings revealed that this model faithfully imitated NAFLD-associated changes. These findings were in accordance with those of other previous studies (*Shirakawa et al., 2011; Zhang & Lu, 2015; Chien et al., 2016; El-Sherbiny et al., 2018* and *Ibrahim & Fathy, 2018*).

Furthermore, this model showed increased serum TNF- $\alpha$  protein and CRP levels, indicating a worsening of the inflammatory burden associated with the NAFLD group.

In this study, oxidative stress biomarkers (MDA) and antioxidant enzymes (GST and SOD) were assessed in rat liver. The current work showed that the antioxidant activities of hepatic SOD and GST significantly decreased and that lipid peroxidation, which contributes to MDA levels, significantly increased in type 2 diabetic rats as reported by *Zhu et al. (2016)*. The increased lipid peroxidation and decreased GST and SOD activities may be due to increased ROS generation (*Diab and Hamza, 2016*).

Our results also revealed that serum irisin levels significantly decreased due to type 2 diabetes mellitus in the NAFLD group compared with the control group. In addition, irisin levels were negatively correlated with BMI, AC, serum glucose, insulin, HOMA-IR index, TG, TC, LDL-c, albumin, ALT, AST, TNF- $\alpha$ , CRP and hepatic MDA levels in the type 2 diabetic group. However, there was significant positive correlation observed with hepatic SOD and hepatic GST.

These findings were supported by previous studies showing that serum irisin levels were gradually reduced with

increased NAFLD severity (*Zhang et al., 2013* and *Polyzos et al., 2014*). However, in contrast to our findings, serum irisin levels were found to be higher in the NAFLD group than in controls (*Choi et al., 2014*). Hyperinsulinemia, insulin resistance, hyperglycemia and high intrahepatic triglyceride content may result in reduced serum irisin levels. These findings are supported by other studies (*Zhang et al., 2013; Al-Daghri et al., 2014; Kuzmicki et al., 2014; Yan et al., 2014* and *Zhang et al., 2014b*), indicating that irisin may act as a protective agent against NAFLD.

The effect of irisin administration on NAFLD in the HFD/STZ-induced type 2 diabetic rat model was examined in this study. Irisin produced a significant decreases in BMI, AC, FBG (fasting blood glucose) levels, HOMA-IR, TG, TC, and LDL-c and increase in HDL-c, in type 2 diabetic rats, indicating improvement of glycemia, lipid control and insulin sensitivity. These findings provided a basis for irisin to be used as a potential promising agent for the management of insulin resistance, hyperlipidemia, and type 2 DM.

Skeletal muscles act as an endocrine organ, secreting myokines that mediate the beneficial effects of exercise (*Pedersen and Febbraio, 2012*). In this situation, irisin, as a myokine, may encourage browning of white adipose tissue, resulting in increased energy expenditure by up regulating the genes associated with browning, such as uncoupling protein 1 (UCP-1) and peroxisome proliferative activated receptor- $\gamma$  (PPAR- $\gamma$ ) (*Boström et al., 2012* and *Zhang et al., 2016*). This process may

be mediated by phosphorylation of p38 mitogen-activated protein kinase and extracellular signal-related kinase (*Zhang et al., 2014a*). Moreover, lipolysis-related genes are up regulated by irisin, increasing the release of glycerol and decreasing lipid deposition in fat cells (*Gao et al., 2016*). Furthermore, irisin increases insulin-mediated glucose uptake by elevating GLUT-4 expression in fat cells (*Huh et al., 2014*). These remarkable properties of irisin make this adipomyokine a potential therapeutic candidate for obesity and other metabolic disorders.

Irisin may prevent liver triglycerides deposition by stimulating the PPAR $\alpha$  signaling pathway involved in lipid metabolism controlling fat oxidation (*Hondares et al., 2011*). In addition, irisin inhibits cholesterol transport and elevates HDL levels (*Xu et al., 2009* and *Badimon and Vilahur, 2012*). Therefore, irisin could play an important role in the prevention of hepatic steatosis.

Regarding the effect of irisin on liver function, its administration resulted in decreased liver weight, serum liver enzymes (ALT and AST) levels, and increased serum albumin level, indicating improvement in liver dysfunction induced by HFD/STZ in NAFLD rat models.

In accordance, the hepatic histopathological examination in the NAFLD group treated with irisin showed amelioration of the degree of fatty infiltration, foci of inflammatory infiltration and ballooning degeneration. Indeed, the current results demonstrated that irisin may prevent the progression of NAFLD in type 2 diabetic rats by

modulating lipid metabolism and dyslipidemia.

The current results indicated that irisin lessened the inflammatory burden in rats with NAFLD and decreased serum TNF- $\alpha$  protein and CRP levels, suggesting a potential anti-inflammatory role of irisin. The present findings were consistent with those of other studies that reported a potential anti-inflammatory effect of irisin (Choi *et al.*, 2014; Polyzos *et al.*, 2014 and Park *et al.*, 2015). In agreement, irisin treatment reduces the inflammatory markers associated with palmitic acid-induced lipogenesis. This decrease may be mediated by inhibition of protein arginine methyl transferase 3 (PRMT3), which is involved in liver lipogenesis (Park *et al.*, 2015). Moreover, irisin may induce phenotypic switching of macrophages from the pro-inflammatory (M1) to anti-inflammatory (M2) state in adipose tissue (Dong *et al.*, 2016). Therefore, serum irisin levels are significantly elevated in the early stages of NAFLD, probably as a defense mechanism, and are reduced with disease progression (Choi *et al.*, 2014).

In addition, the current study demonstrated an antioxidant effect of irisin in rats with NAFLD, as irisin treatment reduced lipid peroxidation contributing to hepatic MDA levels and increased hepatic SOD and GST. Accumulating evidence highlights the antioxidant effect of irisin, as Park *et al.* (2015) showed that irisin diminished FFA-induced oxidative stress via Protein arginine N-methyltransferase 3 (PRMT3) regulation confirmed by inhibiting the expression of inflammatory markers. PRMT1 and PRMT3 share the pathophysiology of hepatic lipogenesis.

Thus, PRMT isoforms could be therapeutic targets. In accordance, the antioxidant activity of irisin has been demonstrated by other studies (Lu *et al.*, 2015; Zhu *et al.*, 2015 and Liu *et al.*, 2017). Therefore, irisin may be a promising agent for the management of diabetic vascular complications (Lu *et al.*, 2015). Additionally, it decreases the oxidative stress induced by high sugar and high fat in human umbilical vein endothelial cells by impeding the activation of PKC-/NADPH oxidase and NF-B/iNOS signaling pathways (Liu *et al.*, 2017).

Moreover, immunohistochemical findings in our study showed strong caspase 3 expression in rats with NAFLD which is in agreement with El-Sherbiny *et al.* (2018). However, after irisin treatment a marked decrease in caspase-3 expression was found, which is in line with Song *et al.* (2014) who reported that irisin may have anti-apoptotic effects via a reduction in the expression of many apoptotic markers induced by hyperglycemia, such as Caspase-3.

Consistent with these findings, Bi *et al.* (2019) demonstrated that exogenous irisin alleviates liver ischemia-reperfusion (I/R) injury. Irisin improved liver function, reduced liver necrosis and cell apoptosis, and relieved the inflammatory response after hepatic I/R. Moreover, it inhibited mitochondrial fission-related protein-dynamamin related protein 1 (drp-1) and fission 1 (Fis-1) expression in hepatic I/R. Additionally, irisin increased mitochondrial content and increased expression of mitochondrial biogenesis-related PPAR $\gamma$  co-activator 1 $\alpha$  (PGC-1 $\alpha$ ) and mitochondrial transcription factor

(TFAM). Furthermore, irisin decreased oxidative stress by up regulating uncoupling protein 2 (UCP-2) expression in hepatic I/R. These results reveal that treatment with exogenous irisin alleviated hepatic I/R injury by restraining mitochondrial fission, promoting mitochondrial biogenesis and relieving oxidative stress. Irisin treatment also appears to be a promising therapeutic approach for hepatic I/R injury.

**Conclusion:** Taken together, these data implied that irisin may be used as a therapeutic agent against the progression of NAFLD in type 2 diabetic rats through its role in glucolipid metabolism and its anti-inflammatory and antioxidant effects confirmed by partial amelioration of hepatic functions and morphology. Furthermore, although of the administration of exogenous irisin achieved beneficial effects on NAFLD in type 2 diabetic rats, the present findings were based only on basic research, and clinical studies are necessary in the future.

Future studies should evaluate the impact of irisin on the hepatic mRNA levels of nuclear factor, erythroid 2-like 2 (Nrf2).

Some of the discrepancies found between the current results and other studies may be due to variations in the animal models, drug doses, and treatment durations.

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## "ايريسين" عامل علاجي واعد محتمل ضد مرض الكبد الدهني غير الكحولي في الجرذان المصابة بالنوع الثانى من مرض السكري

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**خلفية البحث:** يرتبط مرض الكبد الدهني غير الكحولي بقوة بالسمنة ومقاومة الإنسولين ومرض السكري من النوع الثانى.

**الهدف من الدراسة:** هدفت الدراسة الحالية إلى تقييم الآثار المفيدة المحتملة للايريسين على وظائف الكبد في الجرذان البيضاء المصابة بالنوع الثانى من السكرى ومحاولة توضيح الآليات الأساسية.

**مواد وطرق البحث:** شملت الدراسة ثلاثين من ذكور الجرذان البيضاء البالغين والتي وزعت إلى ثلاث مجموعات متساوية: المجموعة الأولى تشمل الجرذان العادية (المجموعة الضابطة)؛ والمجموعة الثانية التي تحوى الجرذان المصابة بالكبد الدهني غير الكحولي جراء الاصابة بالنوع الثانى من مرض السكرى، والمجموعة الثالثة هي الجرذان المصابة والتي تلقت العلاج بالايريسين (0.5 ميكروجرام لكل جرام من وزن الجسم يوميا لمدة 8 أسابيع)

وقد تم استحداث هذا النموذج المصاب بالكبد الدهني بتناول الجرذان لنظام غذائي غني بالدهون لمدة 12 أسبوعاً، ثم حقنهم بجرعة واحدة من الستيروئيد توستون (35 ملجم لكل كجم من وزن الجسم)

و عند نهاية التجربة تم فحص الجرذان فى المجموعات الثلاثة من حيث وزن الجسم، وحساب مؤشر كتلة الجسم، ومحيط البطن، و وزن الكبد، والجلوكوز في الدم، و الإنسولين، و الكوليسترول الكلي، والدهون الثلاثية، و الكوليسترول منخفض الكثافة و الكوليسترول عالي الكثافة. كما تم تقييم مقاومة

الإنسولين ، و قياس إنزيمات الكبد ( الألبومين أمينوترانسفيراز ، أسبارتات أمينوترانسفيراز ) ، والألبومين ، و قياس ايريسين فى الدم ، ومستويات البروتين التفاعلي. علاوة على ذلك ، فقد تم تقييم أنشطة بعض مضادات الأكسدة فى الكبد (سوبرأكسيد ديزميتاز ، والجلوتاثيون اس ترانسفيراز)، كما تم الفحص المعملى للأنسجة الكبدية.

**النتائج : 1-** كشفت النتائج حدوث انخفاض فى مستوى ايريسين فى مصل الدم فى جرذان المجموعة الثانية مع وجود علاقة ارتباط سلبى ذى دلالة احصائية بين هذا الهرمون و كل من حساب مؤشر كتلة الجسم ، و محيط البطن ، والجلوكوز فى الدم ، و الإنسولين، و مقاومة الإنسولين ، و الكوليسترول الكلى ، والدهون الثلاثية ، والكوليسترول منخفض الكثافة و الكوليسترول عالى الكثافة ، والألبومين ، و إنزيمات الكبد ( الألبومين أمينوترانسفيراز ، أسبارتات أمينوترانسفيراز ) ، ومستويات البروتين التفاعلي.

و علاقة ارتباط ايجابى ذى دلالة احصائية مع مضادات الأكسدة فى الكبد (سوبرأكسيد ديزميتاز ، والجلوتاثيون اس ترانسفيراز)

**2-** حقن ايريسين للجرذان ذات الكبد الدهنى والمصابة بداء السكرى أدى إلى انخفاض ذو دلالة احصائية فى مؤشر كتلة الجسم ، ومحيط البطن ، والجلوكوز فى مصل الدم ، و الإنسولين ، و الكوليسترول الكلى ، والكوليسترول منخفض الكثافة مع ارتفاع فى مستوى الكوليسترول عالى الكثافة والألبومين والأنشطة المضادة للأكسدة ، كما أثبت الفحص المعملى تحسنا فى الأنسجة الكبدية لدى الجرذان المصابة بالكبد الدهنى.

**الاستنتاج :** يمكن استخدام ايريسين كعامل واعد ضد تدهن الكبد فى الجرذان المصابة بداء السكرى من النوع الثانى ، ربما من خلال التحكم فى أيض الجلوكوز والدهون ، و عبر آثاره المفيدة المضادة للالتهابات والمضادة للأكسدة.