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THE ROLE OF VISFATIN AND CYTOGLOBIN IN OBESE DIABETIC RATS: THE MODULATORY EFFECTS OF RASPBERRY KETONE

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Introduction: *Clinical and epidemiological studies suggest that patients who are overweight or obese are more at risk in developing glucose intolerance (G/I) and insulin resistance (I/R) leading to type 2 diabetes (T2DM) and cardiovascular disease.*

Aim of work: *Assess the dynamic contribution of visfatin in the development of obesity and/or diabetes and demonstrate their possible molecular mechanism(s) from side and from another side, modulate role of Raspberry ketone (RK) as weight management supplement and illustrate their possible molecular mechanism(s).*

Materials and Methods: *Eighty adult rats were divided into eight groups (10 rats for each group, G); G1: Normal Control Group (Normal diet); G2: Diabetic Control Group (received* streptozotocin *35 mg/kg); G3: Obese Control Group (received high fat diet, HFD); G4: Obese Diabetic Control Group, G5: Raspberry ketone Control Group (received 500 mg/kg), G6: Diabetic rats treated with Raspberry ketone; G7: Obese rats treated with Raspberry ketone and G8: Obese Diabetic rats treated with Raspberry ketone to assess the study's aims, their effect was determined on body weight, OGTT, glucose homeostasis (glucose, insulin, HOMA-IR), oxidative stress markers, cytoglobin, visfatin and liver histopathology.*

Results: *RK caused weight loss, corrected the disturbed glucose and insulin homeostasis, Furthermore, RK increased hepatic content of glutathione (GSH), while decreased hepatic content of malonaldialdehyde (MDA). RK also up regulated hepatic protein expression of cytoglobin, while down regulated hepatic mRNA expression of visfatin.*

Conclusion: *This study assessed the involvement of visfatin and cytoglobin in obese diabetic rats and modulated the role of RK through the efficient rebalance of glucose homeostasis, I/R, the redox status and liver histopathology.*

INTRODUCTION

Obesity is a disorder that affects the balance between energy intake and expenditure. It a serious health problem characterized by an excessive expansion of the white adipose tissue (WAT) coupled with a state of chronic low grade inflammation and oxidative stress (OS)[1].

The incidence of obesity and its related metabolic disorders has increased dramatically in the past decades worldwide. The number of obese individuals worldwide has reached 2.1 billion leading to an explosion of obesity related health problems associated with increased morbidity and mortality[2]. Additionally, 12% of the world adult population currently are considered obese and on the national side 35% of Egypt adults are considered obese. According to the WHO's obesity and overweight fact sheet number 311, obesity worldwide was tripled since 1975[3].

A chronic low-grade inflammation occurring in adipose tissue resulting from chronic activation of the innate immune system is at least in part responsible for obesity-induced insulin resistance (I/R). This adipose tissue inflammation is characterized by changes

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in immune cell populations giving rise to altered adipokine profiles, which in turn induces I/R[4].

Adipo/cytokines are proteins with hormone-like properties mainly produced by adipocytes and released to circulatory system to communicate the functional status of AT with different organs, including brain, liver, pancreas, immune system, vasculature, muscle and AT itself[5].

Visfatin is one of the multifunctional adipokines having variety of function, Recent studies suggested that its over expression possesses a differential role in modulation of OS, inflammation, obesity, IR and DM[6]. There is an evidence that visfatin is significantly increased in adipocytes during the process of adipogenesis, as visfatin has been suggested to be involved in adipocyte differentiation and proliferation. It also promotes the accumulation of fat through the activation of glucose transport and lipogenesis[7].

The mechanism by which visfatin exerts its biological effects are not completely understood. Regarding visfatin role in DM, visfatin has been shown to stimulate myocytes and adipocytes glucose transport and to inhibit hepatocyte glucose production[8].

Cytoglobin is a redox-sensitive protein which has been reported to have a great role in the regulation of oxidative stress, inflammation and fibrogenesis[9]. El-Moselhy *et al.*[10] reported the anti-fibrotic effect of cytoglobin on liver both *in-vivo* and *in-vitro* through modulation of apoptosis. Furthermore, absence of cytoglobin in aged mice was reported to promote multiple organ abnormalities via oxidant/anti-oxidant imbalance and nitric oxide derangement[11]. Also, other authors proven that the deficiency of cytoglobin during cholestasis enhanced liver injury and fibrogenesis[12].

Raspberry ketones (4-(4-hydroxyphenyl) butan-2-one) (RK) are the major aromatic compounds found in red raspberry (Rubus idaeus)[13]. It was suggested that adding RK supplements to weight lowering products may increase the ability of the body to burn fat and may also decrease the accumulation of fat under the skin and around the abdominal organs. RK were postulated to accelerate lipolysis and increase translocation of hormone sensitive lipase (HSL) to the lipid droplets[14].

MATERIALS AND METHODS

Animals

Adult male Wister albino rats with initial body weight 200±15 g were used in the experimental work. Rats were obtained and maintained in the animal house of Faculty of Medicine, Assiut University, under standard laboratory conditions (light/dark (12/12 hrs) cycle, 21±2°C with relative humidity 55%) and were allowed free access to standard rat pellets and tap water ad libitum. All the animal experiments were conducted in accordance with the guide for the care and use of laboratory animals of the National Institutes of Health 1985.

Preparation of normal and high fat diet (HFD)

Normal rat chow was prepared according to Kim *et al.*[15]. It comprised of 65% carbohydrate (60% starch & 5% sucrose), 5% fat (soya bean oil), 20% protein (casein), 5% vitamins and minerals, 5% fiber (barn) and metabolic energy of this diet is 2813 kcal/kg with 8% from fat.

High Fat Diet (HFD) was prepared by following the method of Khalifa *et al.*[16] HFD comprised of 7% wheat flour, 4% bran, 30% casein, 10% glucose, 6% common salt, 3% vitamin mixture and 40% raw melted beef fat to make palettes. Almost 54% of the calories from this diet were from fat contents[16].

High fat diet / streptozotocin T2DM model

High fat diet / streptozotocin (HFD/STZ) T2DM model mimicking human T2DM was designed according to Luo *et al.*[17], After 4 week HFD, the obesity model was considered complete when the average weight of the rats fed HFD was greater than 20% that of the rats fed a normal diet. Briefly rats were fed on HFD for 4 weeks and injected a single low dose of STZ (35 mg/kg i.p. in 0.1 M citrate buffer pH 4.5) purchased from Sigma-Aldrich, MO, USA and continued feeding on HFD for next 4 weeks[17]. HFD for 8 weeks has been used to model the I/R that mimics human T2DM. A single low dose (i.p. 35 mg/kg b.w.) of STZ did not destroy all the beta cells but killed some of them to render the rats become hyper-glycemic[18&19].

Blood glucose level (BGL) was regularly monitored via tail vein using commercially available glucometer. The animals having blood glucose level more than 250 mg/dl & persist for 1week were involved in the experiment. All the animals (groups 2, 4, 6 and 8) used in the experiment developed T2DM and had BGL ≥250 mg.

Experimental design

- Group 1: Normal control Group (NC): 10 rats injected once with ice cold 0.1 M citrate buffer (pH 4.5).
- Group 2: Diabetic Control Group (DC): 10 rats were received STZ.
- Group 3: Obese Control Group (OC): 10 rats were fed with HFD.
- Group 4: Obese Diabetic Control Group (ODC): 10 rats were fed with HFD & were received STZ.
- Group 5-8: 10 rats were administrated RK 500 mg/kg b.w. using oral tubes along EP daily for 8 weeks.
- Group 5: Raspberry ketone Negative Control Group (RKC): 10 rats injected once with ice cold 0.1M citrate buffer (pH 4.5).
- Group 6: Raspberry ketone Diabetic Group (RK+D): 10 rats were received STZ.
- Group 7: Raspberry ketone Obese Group (RK+O): 10 rats were fed with HFD.
- Group 8: Raspberry ketone Obese Diabetic Group (RK+OD): 10 rats were fed with HFD & were received STZ.

Samples and measure parameters

1- Collection of serum and oral glucose tolerance test

At the end of the experiment, blood sample was collected after overnight fasting under inhalation of 2% ether anesthesia by retro-orbital puncture method. These samples were used for measuring the fasting glucose & insulin.

All animals were sacrificed after anesthetization with ether and blood samples were withdrawn from superior vena cava. These samples were collected into centrifuge tubes and left to stand at 4°C for 60 min, then centrifuged at 3000 rpm for 10 min. The sera was isolated and stored at -20°C until determination of the biochemical parameters.

2- Collection of tissues

Liver tissue was dissected into 4 parts and each part was used for different purpose. One part was stored in formalin (10%) and subjected for histopathological examination, The second part was frozen stored for oxidative stress detection and The third and the fourth parts were instantly flash frozen in liquid nitrogen and stored separately at –70°C for subsequent western blotting and qRT–PCR assays.

Metabolic indices

1- Body weight (BW)

The body weight of each animal was measured on day 1, then BW was recorded weekly to follow up the progress of weight. Body weight gain was measured as index of obesity. Percent of changes in body weight were calculated as following:

$$\%BW = (BW\ gain/\ BW) \times 100$$

2- Homeostasis model assessment of insulin resistance (HOMA-IR)

Blood glucose at zero time was assayed by the glucose oxidase method while insulin levels were measured using an ELISA kit. HOMA-I/R was calculated as following:

$$HOMA\text{-}IR = \text{fasting insulin } [\mu U/ml] \times \text{fasting glucose } [mg/dl])/405$$

3- Oral glucose tolerance test (OGTT)

Glucose (200 g/l) was administered orogastrically using a stainless steel gavage needle in a dose of 2 g/kg b.w. Blood samples from supra-orbital sinus & urine samples were collected at 0, 30, 60 and 120 min after glucose administration.

Biochemical measurements

1- Estimation of blood glucose

Blood glucose levels were measured using commercially available glucometer (On Call EZ II ACON laboratories, Inc., USA) from the tail vein.

2- Determination of insulin

Insulin ELISA kit (DRG international Inc., NJ, USA) was used to determine fasting insulin.

3- Measurement of insulin signaling pathway (p-IRS-1/ p-Akt/ GLUT-4)

The liver tissue homogenate was processed as described by the manufacturer using the corresponding ELISA kits; p-IRS-1 (MyBioSource Inc., CA, USA; Cat # MBS9327188); p-Akt (DRG international Inc., NJ, USA, Cat # EIA-3997) and GLUT-4 (Elabscience Biotechnology Co., Ltd, TX, USA; Cat # E-EL-R0430).

4- Measurement of glutathione (GSH) and malonaldialdehyde (MDA) in liver tissue

Glutathione (GSH; Biodiagnostic, Cat # GR 2511) and malonaldialdehyde (MDA; Biodiagnostic, Cat # MD 2529) were determined using biochemical kits.

5- Estimation of cytoglobin protein expression by Western blot

The protein levels of cytoglobin in liver tissue before and after treatment with RK were analysed with a Western blot technique. Protein Assay was performed, according to the manufacturer's protocol, to assess the total protein concentration[20].

6- Quantitative real-time PCR analysis

RNA was extracted from liver tissues using TRIzol reagent according to the manufacturer's instructions and then the samples were purified by digesting the residual DNA using DNase I according to the manufacturer's instructions[21]. The complementary DNA was synthesized using high-capacity cDNA reverse transcription kits according to the manufacturer's instructions. cDNA was then subjected to quantitative real-time PCR amplification using SYBR Green premix Taq. Reactions were run in a real-time PCR system. Relative gene expression levels were calculated using the method of (2-ΔΔCt) using the expression of the beta-actin gene as an internal control. The forward and reverse primers are listed in table 1.

7- Histopathological studies

Specimens from the liver were collected and fixed in neutral buffered formalin 10%, routinely processed and embedded in paraffin wax. Sections of 4-5 um thickness were prepared and stained with Haematoxylin and Eosin for histopathological examination by light microscope (Olympus BX50, Japan). Histopathological alterations were graded as (0) indicated no changes, (1), (2) and (3) indicated mild, moderate and severe alterations, respectively, while the grading was determined by percentage as follows: (<30%) showed mild changes, (<30%–50%) indicated moderate changes and changes more than 50% (>50%) indicated severe changes[22].

8- Statistical analysis

Data were expressed as the mean ± standard error of the mean (SEM). Variables were compared using one way analysis of variance (ANOVA) followed by Tukey's test as appropriate. The *p* value less than 0.05 was considered to be significant. Data analysis was accomplished using Statistical Package for Social Society (SPSS) software program (version 20). Pearson Correlation was estimated to find out the association between adipokines and some related biomarkers in all groups, where (p) and (r) values were recorded.

RESULTS AND DISCUSSIONS

Result

Effect of raspberry ketones on body weight (BW)

Feeding NC and STZ groups with non fat diet (NFD) for 8 weeks, resulted in a significant increase in their BW ($p< 0.05$) as compared to their initial values. Meanwhile, feeding HFD and HFD/STZ groups with HFD for 8 weeks, resulted in significant increase in their body weight ($p< 0.01$) as compared to their initial values suggesting induction of obesity in these groups. In contrast, BW of

Table 1: The forward and reverse primers.

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
Visfatin	CCTCTTGAATTGCTCCTTCA	CCGTATGGAGAAGATCATGG
β-actin	CCACCATGTACCCAG GCATT	ACGCAGCTCAGTAACA GTCC

RKC, RK/STZ, RK/HFD and RK/HFD/STZ groups received RK for 8 weeks, were markedly decreased starting from 1st week and the maximum significant reduction was found after the 8th week ($p< 0.05$, $p< 0.05$, $p< 0.05$ & $p< 0.01$) respectively as compared to the corresponding control groups, the results are illustrated graphically in figures 1&2.

Effect of raspberry ketone on oral glucose tolerance test (OGTT)

As a confirmation of the glucose intolerance (G/I) /insulin resistance (I/R) status, After overnight (16 hrs) fasting, oral administration of glucose solution (2 g/kg BW) produced a significantly increase in glycemia in all groups. In NFD group, the maximum elevation in BGL was observed at 30 min after glucose load and declined to near basal level at 120 min where in HFD/STZ group, the peak increase in BGL was noticed even after 60 min and remained high over the next 60 min with slight decrease. Meanwhile, fasting serum glucose (FSG) in the HFD/STZ group were much higher than that in the normal group at 0, 30, 60, 90 and 120 min by (204%, 134%, 266%, 255% and 227%) respectively. In contrast, RK treated groups, were more sensitive to the glucose challenge as they elicited a highly significant decrease in BGL at 30 min and beyond when compared with their control groups, the results are illustrated graphically in figure 3.

Effect of raspberry ketone on glucose homeostasis-related parameters

After overnight (16 hrs) fasting, the fasting serum glucose (FSG) (zero min) were significantly greater in the STZ, HFD and HFD/STZ control groups ($p< 0.01$) as compared to the NFD control group. In contrast, FSG level of RKC, RK/STZ, RK/HFD and RK/HFD/STZ groups received RK, evident significant decrease ($p< 0.05$, $p< 0.05$, $p< 0.05$ & $p< 0.01$) as compared to the corresponding control groups.

Fasting serum insulin (FSI) were significantly higher in the STZ, HFD and HFD/STZ control groups ($p< 0.01$) as compared to the NFD control group. In contrast, FSI of RKC, RK/STZ, RK/HFD and RK/HFD/STZ groups received RK for 8 weeks, exhibited significant reduction in serum insulin level ($p< 0.05$, $p< 0.05$, $p< 0.05$ & $p< 0.01$) as compared to the corresponding control groups.

Homeostasis model assessment of insulin resistance (HOMA-I/R) of HFD control group was 4.64 times higher than that of NFD group. The significant increase in HOMA-I/R in the STZ, HFD and HFD/STZ groups, indicated the development of I/R ($p< 0.01$) as compared with the NFD control group. In contrast, the I/R of RKC, RK/STZ, RK/HFD and RK/HFD/STZ groups treated with RK for 8 weeks, were significantly reduced ($p< 0.05$, $p< 0.05$, $p< 0.05$ & $p< 0.01$) as compared to the corresponding control groups, the results are illustrated graphically in figure 4.

Effect of Raspberry ketone on liver contents of oxidative stress

The HFD/STZ T2DM model significantly decrease GSH in the STZ, HFD and HFD/STZ control group ($p< 0.01$) while increase MDA content ($p< 0.01$) as compared to the NFD control group. Treatment with RK, significantly increase GSH ($p< 0.05$, $p< 0.05$, $p< 0.05$ & $p< 0.01$) while decrease MDA by ($p< 0.05$, $p< 0.05$, $p< 0.05$ & $p< 0.01$) in RKC, RK/STZ, RK/HFD and RK/HFD/STZ groups as compared to corresponding control groups, the results are illustrated graphically in figure 5.

Effect of Raspberry ketone on hepatic expression of cytoglobin

The HFD/STZ T2DM model significantly down-regulated the hepatic expression of cytoglobin in the STZ, HFD and HFD/STZ control group by (64.35%, 66.96% and 69.14%) respectively as compared to the NFD control group. Meanwhile, treatment with RK, significantly up-regulated the hepatic expression of cytoglobin in RKC, RK/STZ, RK/HFD and RK/HFD/STZ groups by (31.65%, 17.33%, 14.39% and 41.07%) respectively as compared to the corresponding control groups, The results are illustrated graphically in figure 6.

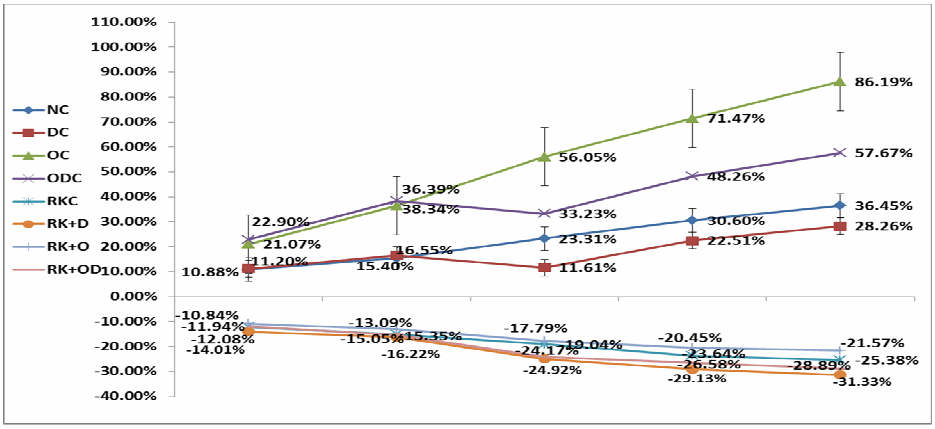

Fig. 1: Effect of Raspberry ketone on % Body weight.

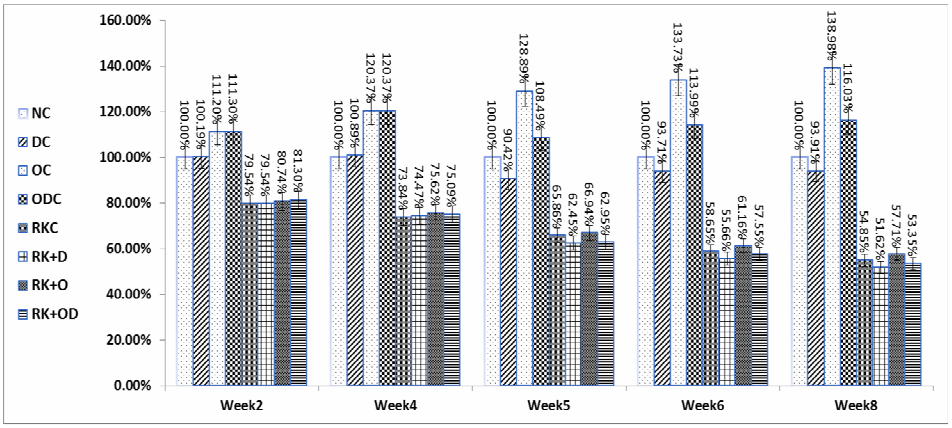

Fig. 2: Effect of Raspberry ketone on % Body weight.

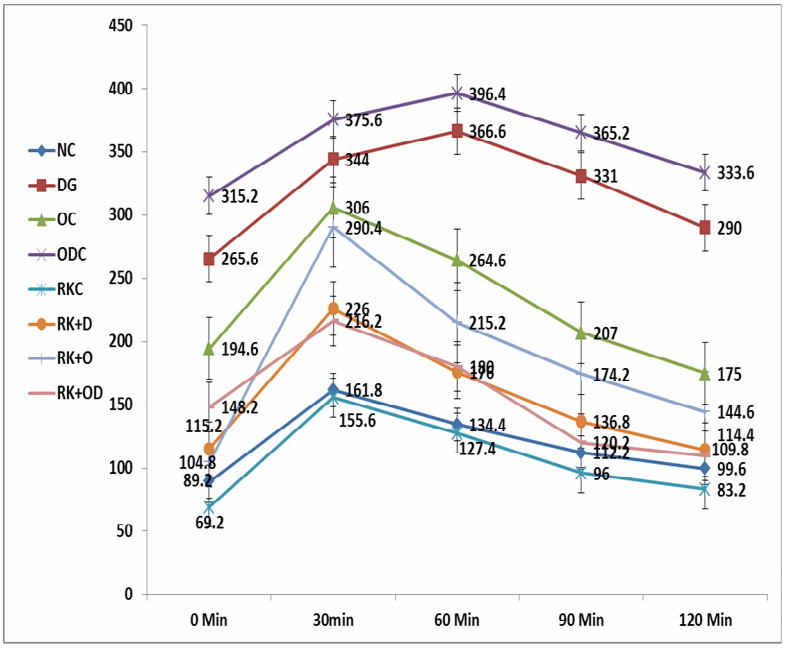

Fig. 3: Effect of Raspberry ketone on Oral Glucose Tolerance Test.

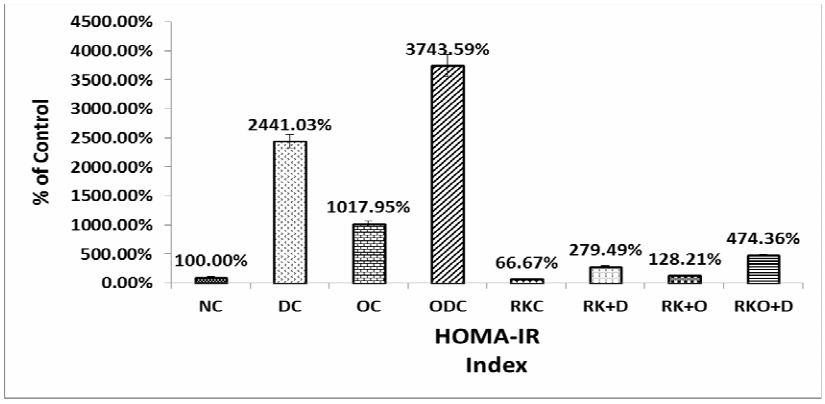

Fig. 4: Effect of RK on HOMA-IR in HFD/STZ T2DM rats.

Data are presented as means ± SEM (*n*= 10 animals/group). Statistical analysis was carried out using one-way ANOVA followed by unpaired Student's t-test or Tukey's Multiple Comparison test.

RKCG vs NCG (significant); RK+D, DC vs NCG (highly significant); RK+O, OC vs NC (highly significant); RK+OD, ODC vs NC (highly significant).
(HOMA-I/R) homeostasis model assessment of insulin resistance.

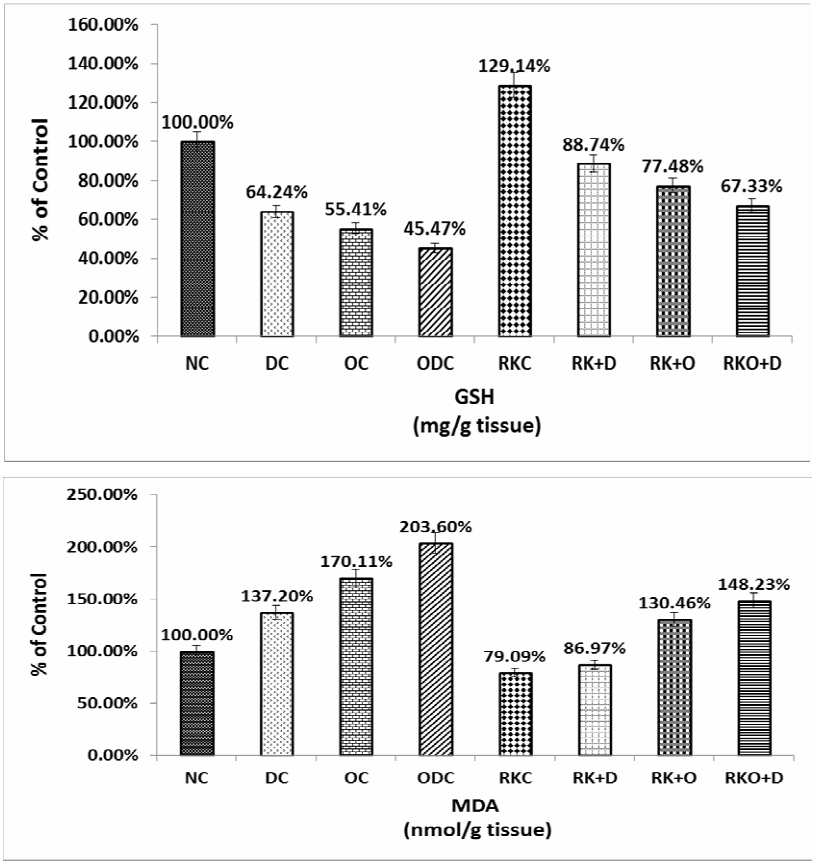

Fig. 5: Effect of Raspberry ketone on liver contents of GSH & MDA.

Data are presented as means ± SEM (*n*= 10 animals/group). Statistical analysis was carried out using one-way ANOVA followed by unpaired Student's t-test or Tukey's Multiple Comparison test.

RKCG vs NCG (significant); RK+D, DC vs NCG (significant); RK+O, OC vs NC (significant); RK+OD, ODC vs NC (significant).

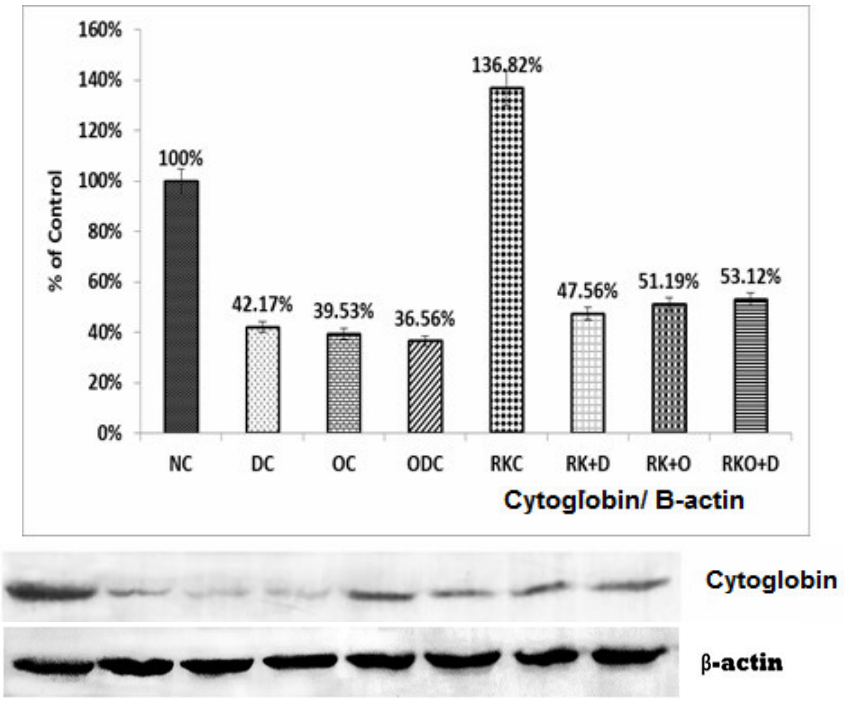

Fig. 6: Effect of Raspberry ketone on hepatic expression of cytoglobin / β-actin.

Data are presented as means ± SEM (*n*= 10 animals/group). Statistical analysis was carried out using one-way ANOVA followed by unpaired Student's t-test or Tukey's Multiple Comparison test.

RKCG vs NCG (significant); RK+D, DC vs NCG (significant); RK+O, OC vs NC (significant); RK+OD, ODC vs NC (significant).

The role of visfatin on insulin signaling transduction pathway (IRS-1 /AKT/GLUT4) and the effect of raspberry ketone in HFD/STZ T2DM rats

The HFD/STZ T2DM model significantly up-regulated the liver expression of visfatin in the STZ, HFD and HFD/STZ control groups by (153.33%, 270.35% and 487.26%) respectively which leading to I/R and altered signaling transduction pathway as follows, I/R inactivated p-IRS-1, which in turn inactivated both p-AKT and GLUT-4 by (61.30%, 68.91% and 79.86%); (61.19%,75.59% and 86.90%) & (77.67%, 83.48% and 88.95%) denoting a state of I/R, respectively as compared to the NFD control group.

Treatment with RK, significantly down-regulated the liver expression of visfatin in the RKC, RK/STZ, RK/HFD and RK/HFD/STZ by (40.00%, 69.36%, 75.31% and 88.68%) respectively, which reverted the deleterious effects of I/R and enhanced the signaling transduction pathway as follows, RK increased p-IRS-1 by (10.23%, 50.61%, 53.32% and 95.28%), which in turn activated both p-AKT by (25.40%, 199.51%, 179.28% and 317.57%) and GLUT-4 by (20.80%, 193.05%, 259.49% and 346.20%) respectively as compared to the corresponding control groups, the results are illustrated table 2 and graphically in figure 7.

Effect of Raspberry ketones on liver histopathological changes

Microscopically, liver of NCG revealed the normal histological structure of hepatic lobule, from central vein and concentrically arranged hepatocytes in hepatic cords (Fig. 8a). On the contrary, liver of DCG showed variable histopathological alterations described by hepatocellular vacuolar degeneration, focal hepatic necrosis associated with inflammatory cells infiltration (Fig. 8b), portal infiltration with inflammatory cells and fibroplasia in the portal triad around the bile duct. On the other hand, liver of OCG revealed microvesicular and macrovesicular steatosis of hepatocytes, congestion of central veins (Fig. 8c) and hepatic sinusoids. Focal hepatocellular necrosis

Table 2: Pearson correlation analysis among visfatin & its related parameters.

	Dependent variable	R	p-value
Independent variable Visfatin	p-IRS-1	-.644**	0.000
	p-AKT	-.797**	0.000
	GLUT-4	-.754**	0.000
	Insulin	0.876**	0.000
	HOMA-IR	0.891	0.000
	%BW	0.616	0.000

The liver content of visfatin showed:

- Negative association with p-IRS-1.
- Strong negative association with both p-AKT & GLUT-4.
- Strong positive association with insulin, HOMA-IR
- Positive association with % BW.

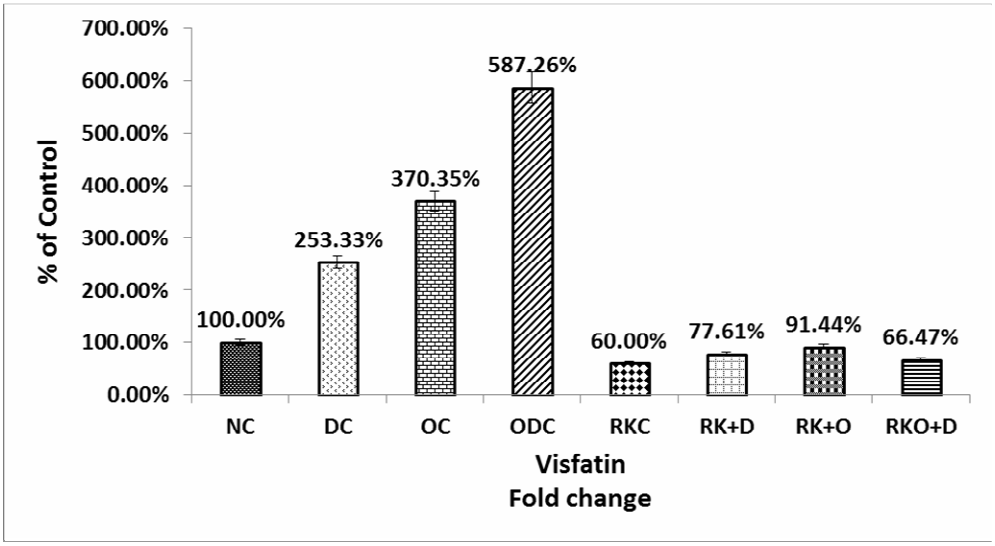

Fig. 7a: The role of visfatin on insulin signaling pathway (PI3K/AKT/GLUT4) and the effect of Raspberry ketone on HFD/STZ T2DM rats.

Data are presented as means ± SEM (*n*= 10 animals/group). Statistical analysis was carried out using one-way ANOVA followed by unpaired Student's t-test or Tukey's Multiple Comparison test.

RKC vs NC (significant); RK+D, DC vs NC (highly significant); RK+O, OC vs NC (highly significant); RK+OD, ODC vs NC (highly significant).

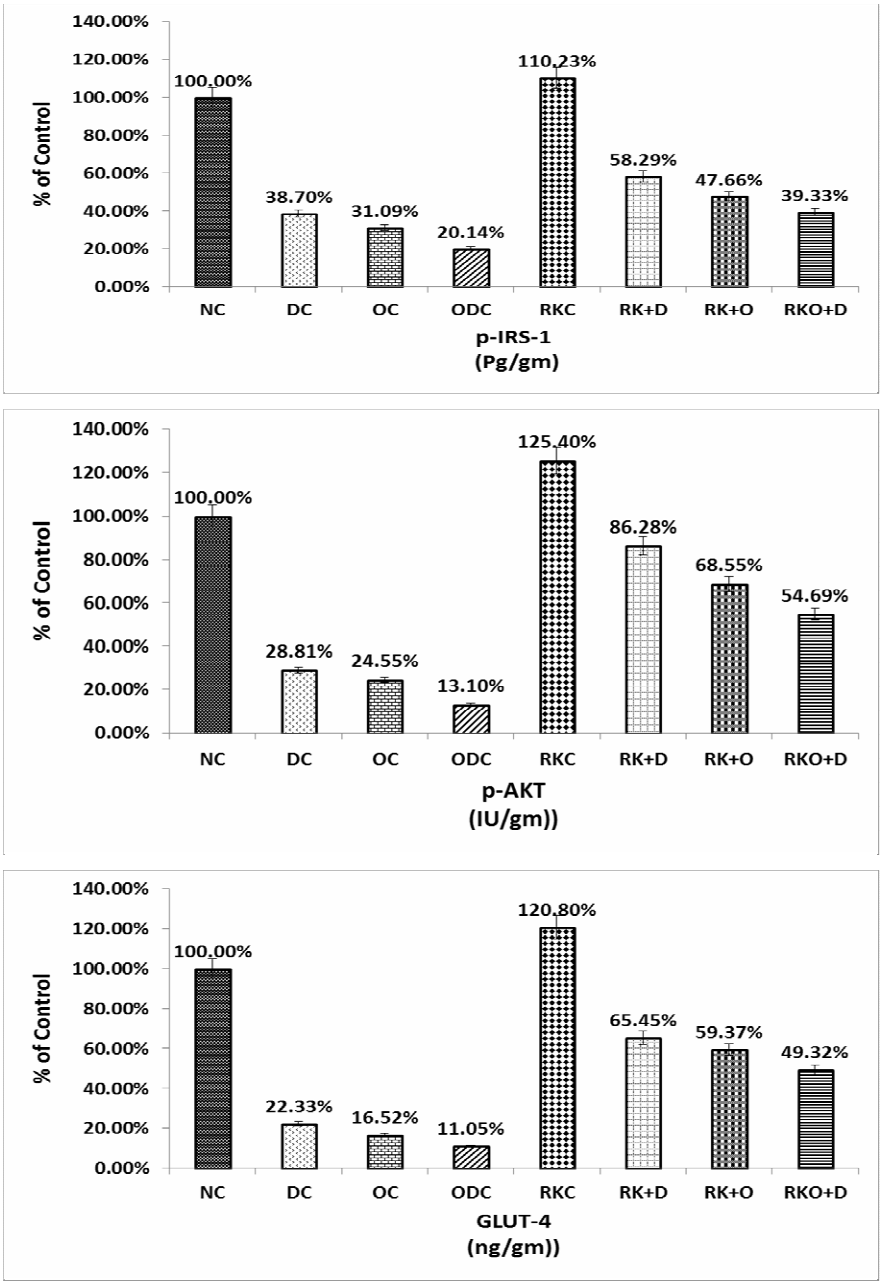

Fig. 7b: The role of visfatin on insulin signaling pathway (PI3K/AKT/GLUT4) and the effect of Raspberry ketone on HFD/STZ T2DM rats.

Data are presented as means ± SEM (*n*= 10 animals/group). Statistical analysis was carried out using one-way ANOVA followed by unpaired Student's t-test or Tukey's Multiple Comparison test.

RKCG vs NCG (significant); RK+D, DC vs NCG (significant);→ RK+O, OC vs NC (significant); RK+OD, ODC vs NC (significant).
(p-IRS-1): phosphorylated insulin receptor substrate; (p-AKT) : phosphorylated protein kinase and GLUT4) : glucose transporter 4.

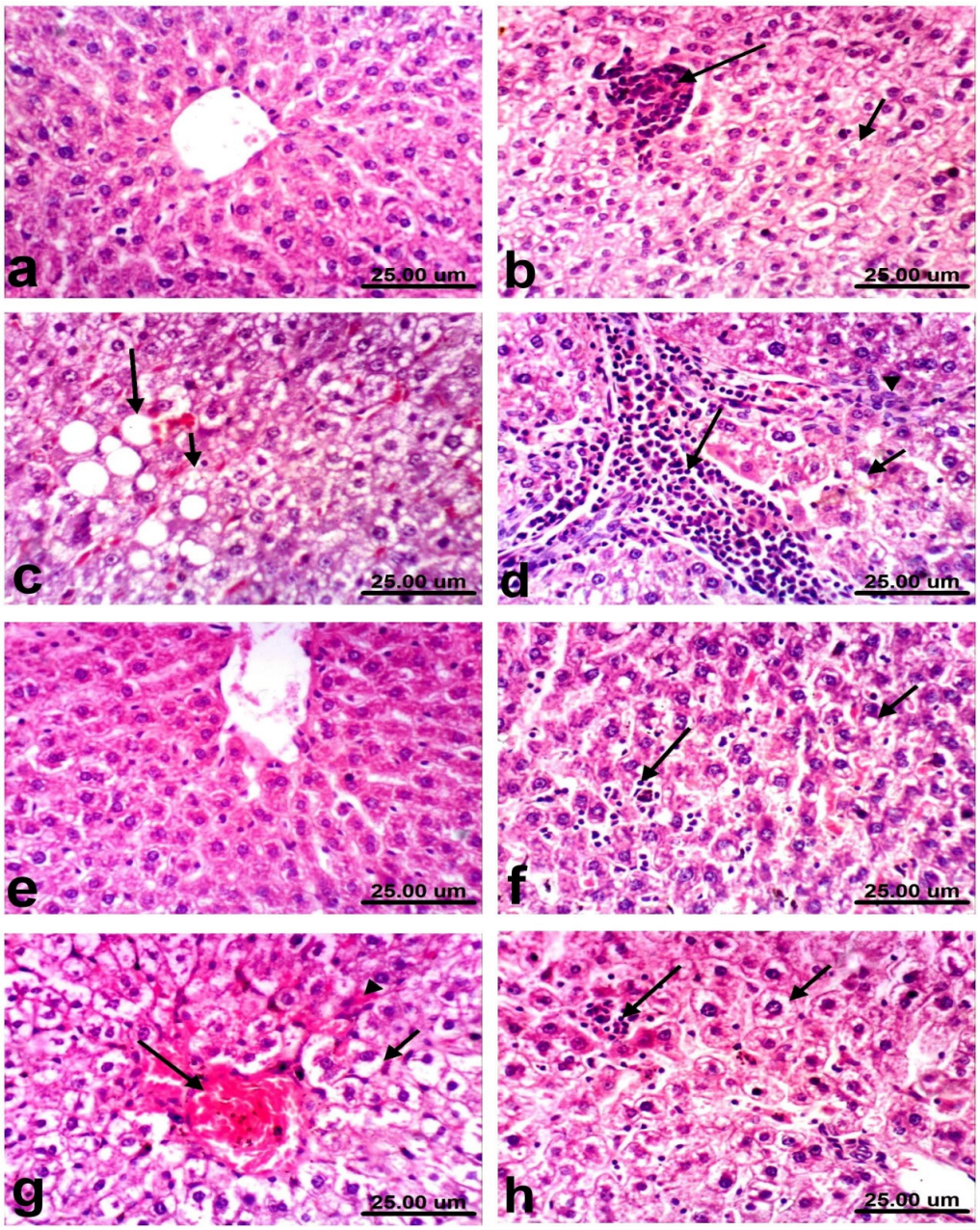

Fig. 8: Histological H&E stained liver sections of rats:

a) NC showing the normal histological structure of hepatic lobule, from central vein and concentrically arranged hepatocytes in hepatic cords. b) DCG showing hepatocellular vacuolar degeneration (short arrow) & focal hepatic necrosis associated with inflammatory cells infiltration (long arrow). c) OCG showing microvesicular (short arrow) and macrovesicular (long arrow) steatosis of hepatocytes as well as congestion of central vein (arrow head). d) ODG showing hepatocellular vacuolar degeneration (short arrow), portal infiltration with inflammatory cells (long arrow) and oval cells proliferation (arrow head). e) RKCG showing no histopathological alterations. f) RK+DG showing vacuolar degeneration of some hepatocytes (short arrow) and sinusoidal leukocytosis (long arrow). g) RK+OD showing hydropic degeneration of hepatocytes (short arrow), congestion of central vein (long arrow) and hepatic sinusoids (arrow head). h) RK+ODG showing slight hydropic degeneration of hepatocytes (short arrow) and small focal hepatocellular necrosis associated with inflammatory cells infiltration (long arrow) (**H & E**, scale bar 25 um).

and apoptosis associated with inflammatory cells infiltration were noticed in some sections. Moreover, severe histopathological alterations were noticed in liver of ODCG. All examined sections from this group showed hepatocellular vacuolar degeneration, focal hepatic necrosis associated with inflammatory cells infiltration, portal infiltration with inflammatory cells and oval cells proliferation (Fig. 8d). Meanwhile, examined sections from RKC revealed no histopathological alterations (Fig.8e). Restoring of the histological structure of hepatic tissue was noticed in liver from RK/DG. Most examined sections from this group showed slight vacuolar degeneration of some hepatocytes and sinusoidal leukocytosis (Fig. 8f). Moreover, marked improvement in the histopathological picture was noticed in the liver of RK/OG, examined sections showed hydropic degeneration of hepatocytes, congestion of central vein and hepatic sinusoids (Fig. 8g). Additionally, all examined sections from RK/ODG showed improved histological picture. Mild histopathological changes were observed and described as slight activation of Kupffer cells, slight hydropic degeneration of hepatocytes and small focal hepatocellular necrosis associated with inflammatory cells infiltration (Fig. 8h).

Histopathological lesions score were summarized in table 3.

Discussion

Visfatin is a highly conserved 52 kDa protein whose biological functions have been under study since its discovery in 1994[5]. Today, it is accepted that it is a multifaceted protein that exists in both intra- and extracellular compartments. It is considered as an important adipokine secreted by visceral adipose tissue (VAT). Several studies have demonstrated its level in obesity and T2DM[4].

The mechanisms by which visfatin exerts its biological effects are not completely understood. Regarding visfatin role in DM, visfatin has been shown to stimulate myocytes and adipocytes glucose transport and to inhibit hepatocyte glucose production[20].

These effects are achieved by direct binding to the insulin receptor (IR), likely in a binding site different from that of insulin, which induces tyrosine phosphorylation of the IR and phosphorylation of insulin receptor substrate-1/2 (IRS-1/2). The subsequent binding of PI3K to IRS-1/2 promotes the activation of PKB/Akt. Moreover, it also helps in translocation of glucose transporter-4 (GLUT4) receptors from the intracellular pool to plasma membranes[21].

Table 3: Histopathological lesions scores of liver (H&E).

Histopathological lesion	NC	DC	OC	ODC	RKC	RK+D	RK+O	RK+OD
Kupffer cells activation	0	3	3	3	0	1	1	2
Hepatocellular vacuolar degeneration or steatosis	0	3	3	3	0	2/1	1	2
Focal hepatic necrosis associated with inflammatory cells infiltration	0	2	2	3	0	0	1	1
Portal infiltration with inflammatory cells	0	3	2	3	0	0	0	1
Fibroplasia in the portal triad	0	2	2	3	0	0	0	1

NC, Normal control; DC, Diabetic control; OC, Obese control; ODC, Obese diabetic control; RKC, Raspberry ketone control; RK+D, Diabetic rats received Raspberry ketone; RK+O, Obese rats received Raspberry ketone; RK+OD, Obese diabetic received Raspberry ketone.

The major pathway by which RK acted to combat obesity was the visfatin/insulin signaling pathway (p-IRS-1/p-AKT/GLUT4), which was inhibited by the HFD to signify the associated I/R and the increased glucose level. Likewise, the deleterious effects of HFD on this signaling pathway was documented by Liu *et al.*[22] where feeding pigs HFD for 6 months caused a significant decrease in signaling pathway expression compared to NFD group[22].

In the current study, RK significantly decreased liver expression of visfatin, which is agreement with other results in adipose tissue (AT) reported by many investigators[11] from side and from another side, RK succeeded to activate/phosphorylate p-IRS-1, which in turn activated its downstream molecule Akt. The activation of this pathway extended to increase GLUT-4 and consequently decreasing both insulin and glucose as well as improving insulin sensitivity (I/S), Our results in liver tissue is agreement with other results in AT reported by many researchers[23].

Concerning the potential clinical value of visfatin as a biomarker, some controversial data can be found. Thus, elevated visfatin levels have been reported in several published articles, including a meta-analysis implemented by Chang *et al.*[24] who showed that the concerning the association between visfatin and overweight/obesity, T2DM & insulin resistance (I/R); By analyzing a total of 62 published articles, these authors concluded that visfatin concentrations were increased. Furthermore, a positive correlation between visfatin levels and I/R was found[24], this supported the present study's finding.

In agreement with our results, more recent study by Jurdana *et al.*[25] who showed that the higher fasting serum visfatin levels in over-weight and obese middle-aged adults compared with normal weight subjects[25] similarly to the results obtained by Legakis *et al.*, in patients with T2DM[26].

In agreement with our results, another recent study has proved that visfatin levels were significantly increased in T2DM compared to healthy subjects; furthermore, higher levels of visfatin were observed in T2DM patients with metabolic syndrome than in those without metabolic syndrome[27]. Despite all the above described, there are conflicting results concerning the disease-associated variation of circulating visfatin levels as other studies have found unchanged or even decreased levels of serum visfatin in these metabolic diseases[28&29], which disagreement with our results.

Visfatin was reported to have a direct relationship with T2DM and to be upregulated in response to hyperglycemia to reduce hepatic gluconeogenesis and stimulate glucose utilization in adipocytes[30]. Visfatin was found to regulate insulin secretion and significantly upregulate the mRNA expression of several key diabetes related genes[31].

In our work, in a stepwise linear regression model contained visfatin applied to the different study groups to evaluate factors affecting HOMA-IR, insulin & %BW. We found that, there were a strong positive association between visfatin and insulin & HOMA-IR. Meanwhile, there was positive association between visfatin and %BW. So our data clearly demonstrated that, visfatin was significantly found to be an independent factor affecting the level of I/R which more tightly correlated with visfatin.

Cytoglobin is anovel liver marker, He *et al.*[32] reported that the anti-fibrotic effect of cytoglobin on liver both *in-vivo* and *in-vitro* through modulation of apoptosis[32]. Furthermore, absence of cytoglobin in aged mice was reported to promote multiple organ abnormalities via oxidant/anti-oxidant imbalance and nitric oxide derangement[33]. Also, other authors proven that the deficiency of cytoglobin during cholestasis enhanced liver injury and fibrogenesis[34].

According to our results, I/R significantly decreased the hepatic protein expression of cytoglobin in the STZ, HFD and HFD/STZ control group as compared to the NFD control group. Meanwhile, treatment with RK reverted the deleterious effects of I/R. RK markedly up-regulated the hepatic protein expression of cytoglobin in RKC, RK/STZ, RK/HFD and RK/HFD/STZ rats as compared to the corresponding controls.

There were previous studies have positively associated both body mass index (BMI) and the state of oxidative stress (OS)[35&36]. Furthermore, impaired redox balance blights the insulin release[37], which can be considered as a cause for I/R in obese individuals. Also, OS could trigger obesity by

stimulating the deposition of WAT and altering food intake; both cell culture and animal studies have demonstrated that OS can cause an increase in pre-adipocyte proliferation adipocyte differentiation and the size of mature adipocytes[38&39].

HFD caused an imbalance to the tissue OS by disrupting its antioxidant defense mechanisms; according to Rosas-Villegas *et al.*[40] rats with HFD and 5% fructose diet caused inflammation, enhanced lipogensis and imbalanced tissue's antioxidant defense mechanisms[40].

The study's data showed that RK have prevented the HFD induced redox imbalance, where they decreased lipid peroxidation product (MDA), while augmented reduced glutathione (GSH) defense molecule. In-line with these current results, earlier studies have verified AOC of RK[41]. Reduced glutathione is a major antioxidant in liver cells. When the balance between oxidant & AO is disrupted, OS is generated[42].

Consistently, HFD was reported to increase OS resulting from pressure of the large body mass[43&44]. Interestingly, it was reported that OS is a key element in pathogenesis of non-alcoholic fatty liver disease[45]. Additionally, HFD results in an increment in hepatic fat accumulation that takes place before significant increment in peripheral fat deposition takes place[46].

In the current study, the HFD/STZ rats showed an increment in their body weight (BW), which is consistent with several previous studies[47-52]. The increased BW could be attributed to the consumption of a diet rich in energy in the form of saturated fats, which deposit in various body fat pads and decrease energy expenditure as compared to the NFD rats[53]. In our results, the administration of RK successfully induced weight loss, Our data are in agreement with previous study demonstrating by Kim *et al.*[54] who showed that the results decreased the body weight gain, leading to body weights near the control group through 8 weeks supplementation[54]. Also, our results are in agreement with the result of several studies[23&43].

In harmony with other results, the HFD/STZ T2DM rats displayed a significant increase in serum glucose & insulin levels as well as HOMA-IR[47,49&50].

Rats suffered from IR as evidenced by the impaired response to the OGTT and the increase in HOMA-IR index along with the reported compensatory hyperinsulinemia. HFD-induced IR could be explained by the glucose-fatty acid (FA) cycle, where the high triglycerides level reported here, could constitute a source of increased FA availability and oxidation. Increased FA oxidation reduces glucose uptake and utilization in skeletal muscle leading to compensatory hyperinsulinemia, a common feature of Srivastava and Apovian[53] & Randle *et al.*[55]. Consequently, IR in the HFD rats would render the rats mildly hyperglycemic, thus enhancing their susceptibility to the diabetogenic effect of STZ. Then severe hyperglycemia was eventually achieved by injecting a subdiabetogenic dose of STZ[47,53&56].

In the present study using RK simultaneously with HFD decreased both insulin and glucose levels along with a decline in IR confirming the results of a previous work on RK against non alcoholic steatohepatitis model[8]. Also in harmony with other studies, our results are in agreement with the results of many investigators[23&43].

The present results indicated that HFD increased HOMA-IR index and high IR in turn, is crucial for development of fatty liver; this agrees with previous reports[11&57]. Also, In our results, the correlation of HOMA-IR from side and OGTT from another side, demonstrated that RK protected animals against the I/R and G/I.

Conclusion

The present study is concluded that, the induction of obesity and/ or diabetes result in chronic low-grade inflammation that manifested by down expression of cytoglobin that distrusted its role in the regulation of oxidative stress, inflammation and fibrogenesis while over expression of visfatin that disturbed insulin signaling pathway (p-IR/p-AKT/GLUT4) from side and from another side induced a significant weight gain, alterations in glucose homeostasis, insulin resistance (I/R). Additionally, result in liver damage and disbalanced the redox status which confirmed by liver histopathologically.

Meanwhile, treatment with RK leads to correct the metabolic state which result in

improvement of inflammation state that manifested by overexpression of cytoglobin that improvement its role in the regulation of oxidative stress, inflammation and fibrogenesis while downexpression of visfatin that regulate insulin signaling pathway (p-IR/p-AKT/GLUT4) from side and from another side RK provides a pronounced loss in body weight, which was confirmed by the drop in %BW and insulin sensitization effect, which was confirmed by the drop in HOMA-IR. Additionally, treatment with RK overcome the liver damage and restores antioxidant enzymes, which confirmed by liver histopathologically.

Recommendation

Our study recommended that, insulin resistance (I/R) is more tightly correlated with visfatin, thus visfatin appeared to be more important determinant of I/R. So, this finding open the door for further studies are needed to additionally clarify other pathways that may be involved in the effect of visfatin on obesity and/or diabetes from side and from another side modulation of visfatin secretion may offer novel approaches in the treatment of a variety of diseases. Also, further studies are needed to additionally clarify other pathways that may be involved in the effect of RK on obesity and/or diabetes from side and from another side RK in combinations are needed to be studied in long term use to assess more its efficacy and safety as weight management supplement.

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دور بروتينات الفسفاتين وسيتوجلوبين في الفئران المصابة بالسمنة والبول السكري: التاثيرات المعدلة لكيتون التوت

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المقدمة: اوضحت الدراسات التطبيقية والتتبعية ان الاشخاص الذين يعانون من زيادة الوزن او السمنة تجعلهم مهددون بحدوث مناعة الجلوكوز ومقاومة الأنسولين مما يمهد لإصابتهم بمرض السكري من النوع الثاني وبعض امراض الاوعية القلبية.

أهداف البحث: دراستنا تهدف إلى التعرف علي دور كلا من الفسفاتين والسيتوجلوبن في مرضي السمنة والسكري وكذلك دراسة المسارات المحتملة لهم من ناحيه ومن ناحيه اخري دراسة دورالمسارات المحتملة لتأثير كينونات الراسبرى (مستخلص من ثمرة التوت الأحمر) على السمنة ومقاومة الأنسولين.

النموذج العملي: تم استخدام ثمانون من الجرذان تم تقسيمهم الي ثمانية مجموعات كل مجموعة تحتوي علي عشرة جرذان:

١. مجموعه تمت تغذية الجرذان فيها بحمية عالية السعرات الحرارية تحتوي على نسبة عالية من الدهون وذلك لتطوير حيوانات مقاومة للأنسولين لمدة ٨ أسابيع متخللا بجرعة صغيرة واحدة من الستربتوزوتوسين (٣٥مجم/كجم) في بداية الاسبوع الخامس (المجموعة الرابعة) والتي استخدمت كمجموعة ضابطه لمجموعه اخري مماثله عولجت بكيتونات الراسبرى (٥٠٠مجم/كجم) (المجموعة الثامنة).

٢. مجموعه تمت تغذيتها الجرذان فيها بحمية عالية السعرات الحرارية تحتوي على نسبة عالية من الدهون وذلك لتطوير حيوانات مقاومة للأنسولين لمدة ٨ أسابيع (المجموعة الثالثة) والتي استخدمت كمجموعة ضابطه لمجموعه اخري مماثله عولجت بكيتونات الراسبرى (المجموعة السابعة).

٣. مجموعه تم اعطاء الجرذان فيها جرعة صغيرة واحدة من الستربتوزوتوسين (٣٥مجم/كجم) في بداية الاسبوع الخامس (المجموعة الثانية) والتي استخدمت كمجموعة ضابطه لمجموعه اخري مماثله عولجت بكيتونات الراسبرى (المجموعة السادسة).

٤. مجموعه تمت تغذية الجرذان (نظرائهم العاديين) فيها بالماء العادي وطعام بلا دهون خارجية ليكون بمثابة المجموعة الضابطة "الكونترول" (المجموعة الأولى) والتي استخدمت كامجموعة ضابطه ايضا لمجموعه اخري مماثله عولجت بكيتونات الراسبرى (المجموعة الخامسة).

لتقييم الآليات المحتملة للمادة محل الاختبار تم قياس عدة مؤشرات في مصل الدم وهي سكر الجلوكوز و الأنسولين و مقدار مقاومة الانسولين. وكذلك تم قياس الفيسفاتين والسيتوجلوبن و مستوى الإجهاد التأكسدى لخلايا الكبد وفحص خلايا الكبد مجهريا.

وامتدادا لما تقدم تم تسجيل ومتابعة تأثير العلاج على وزن الجرذان بوزنهم مرة أسبوعياً و حساب النسبة المئوية التي فقدتها كل مجموعة من وزنها وكذلك تسجيل ومتابعة قياس تأثير العلاج على مستوى سكر الدم بقياسه مرتين اسبوعيا.

النتائج: أظهرت النتائج العملية.قدرة كيتونات الراسبرى علي فقدان واضح في وزن الجرذان المعالجة وتصحيح المخاطر الأيضية للسمنة من خلال احداث تحسن ذا دلالة إحصائية فى توازن الجلوكوز/الأنسولبن فى مصل الدم و تقليل مقاومة الأنسولين وزيادة حساسية الأنسجة للأنسولين. وعلى المستوى الجزيئي الكبدي : اظهرت النتائج تثبيط الفيسفاتين بينما ادت الي تنشيط السيتوجلوبن وكذالك خفض مستوى الإجهاد التأكسدى لخلايا الكبد من خلال زيادة الجلوتاثيون المختزل (GSH) بينما خفض ناتج بروكسيدات الدهون (MDA) واخيرا استطاعت كيتونات الراسبرى تحسين حالة خلايا الكبد التى تم فحصها مجهرياً.

الإستنتاج: اوضحت هذه الدراسه آلية عمل كلا من الفيسفاتين والسيتوجلوبن في الجرذان السمينه السكريه من ناحيه ومن ناحيه اخري اوضحت الية عمل كيتونات الراسبرى من خلال اعادة توازن الجلوكوز ومقاومة الأنسولبن ومستوى الإجهاد التأكسدى وتحسن حالة خلايا الكبد.