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To cite this article: Effat A. Khowailed, Hanan Ahmed Seddiek, Manal Moustafa Mahmoud, Laila A. Rashed & Fatma Elsayed Ibrahim (2018) Effect of metformin on Sirtuin-1 disorders associated with diabetes in male rats, Alexandria Journal of Medicine, 54:4, 373-381, DOI: [10.1016/j.ajme.2017.09.002](https://doi.org/10.1016/j.ajme.2017.09.002)

To link to this article: <https://doi.org/10.1016/j.ajme.2017.09.002>



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Published online: 17 May 2019.



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# Effect of metformin on Sirtuin-1 disorders associated with diabetes in male rats

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## ARTICLE INFO

### Article history:

Received 26 December 2016  
Revised 15 August 2017  
Accepted 18 September 2017  
Available online 5 October 2017

### Keywords:

DM  
Metformin  
SIRT1  
Pancreatic apoptosis

## ABSTRACT

**Background:** Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance, hyperinsulinaemia and hyperglycaemia. Increased glucose production through abnormally elevated hepatic gluconeogenesis is central to the manifestation of hyperglycaemia in T2DM. Metformin corrects hyperglycaemia mainly through inhibition of gluconeogenesis. Sirtuin 1 (SIRT1) has been identified as regulator of gluconeogenic gene expression. The present study aimed to evaluate the effect of metformin on SIRT1 level and activity in liver and pancreas of diabetic rats. Further, the possible role of SIRT1 on metabolic disorders associated with diabetes mellitus, including serum levels of glucose, insulin, triglyceride (TG) and high density lipoproteins (HDL), will be explored.

**Methods:** Thirty-two male albino rats were divided into control group (GpI), diabetic (DM) group (GpII), (metformin + DM) group (GpIII) administered 120 mg/kg metformin daily for 1 month before induction of diabetes, (DM + metformin) group (GpIV) administered 250 mg/kg metformin daily for 1 month after induction of diabetes. At the end of the study, BMI%, serum levels of glucose, insulin, TG and HDL, HOMA, SIRT1 level and activity in liver and pancreas and pancreatic DNA ladder were assessed.

**Results:** Our results showed significant decrease in serum glucose, insulin and TG levels and HOMA; significant increase in HDL level and SIRT1 level and activity in liver and pancreas beside the marked disappearance of pancreatic apoptosis in GpIII & IV relative to GpII. Regarding BMI%, it showed no significant changes in GpIV relative to GpII. No significant change was recorded between GpIII and GpIV regarding all studied parameters except on serum TG.

**Conclusion:** Lowered SIRT1 in diabetes was improved by the administration of Metformin. Consequently, the pathophysiological disorders associated with T2DM were improved.

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Peer review under responsibility of Alexandria University Faculty of Medicine.

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<https://doi.org/10.1016/j.ajme.2017.09.002>

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## 1. Introduction

Prevalence of type 2 diabetes mellitus (T2DM) has increased dramatically over the past four decades. T2DM is characterized by insulin resistance, hyperinsulinemia and hyperglycemia. Also, abnormal elevated hepatic gluconeogenesis is central to the manifestation of hyperglycemia in T2DM.<sup>42</sup>

Beside, an increase in beta cell apoptosis is an important factor contributing to beta cells loss and the onset of T2DM.<sup>46</sup> There is in vitro evidence that gluco-toxicity and lipo-toxicity exert synergistic effects to impair the secretory function of beta cells and to promote apoptosis in T2DM.<sup>16</sup>

Sirtuin 1 (SIRT1): a homolog of the yeast protein Silent Information Regulator 2 (Sir2), encodes the NAD (nicotinamide adenine dinucleotide) dependent histone deacetylase enzyme. It may play a key role in stress responses and cellular metabolism, through deacetylating a variety of substrates, including histones, transcription factors and coregulators, to regulate target gene expression both positively and negatively.<sup>35</sup>

SIRT1 activators improve whole-body glucose homeostasis and insulin sensitivity in adipose tissue, skeletal muscle and liver. Thus, SIRT1 activation is a promising new therapeutic approach for treating diseases of ageing such as T2DM.<sup>35</sup>

Metformin, the primary therapeutic agent for T2DM patients, corrects hyperglycemia and hyperinsulinemia predominantly by enhancing insulin-mediated suppression of hepatic glucose production and enhancing insulin-stimulated glucose uptake by skeletal muscles.<sup>25</sup>

Caton et al.,<sup>9</sup> reported that the activation of hepatic AMPK by metformin will consequently increase SIRT1 activity. This will result in reduced levels and activity of the coactivators cAMP-response element binding protein-regulated transcription coactivator 2 (TORC2) or CRTC2) level – mediated gluconeogenesis, thus lowering plasma glucose and insulin.

In our previous work on similar model, we investigated the possible influence of 30% caloric restriction on SIRT1 in diabetic rats,

and we found that both pre-diabetes CR and post-diabetes CR regimens were associated with significant improvement of insulin resistance and its subsequent hyperglycemia beside their role in attenuation of the marked decrease of SIRT1 in the liver and pancreas that accompanied the induction of type 2 diabetes mellitus.<sup>18</sup>

In the present study, we investigated the effect of metformin on SIRT1 level and activity in liver and pancreas of diabetic rats. Further, the possible role of SIRT1 on metabolic disorders associated with diabetes mellitus will be explored.

## 2. Material and methods

### 2.1. Animals and experimental design

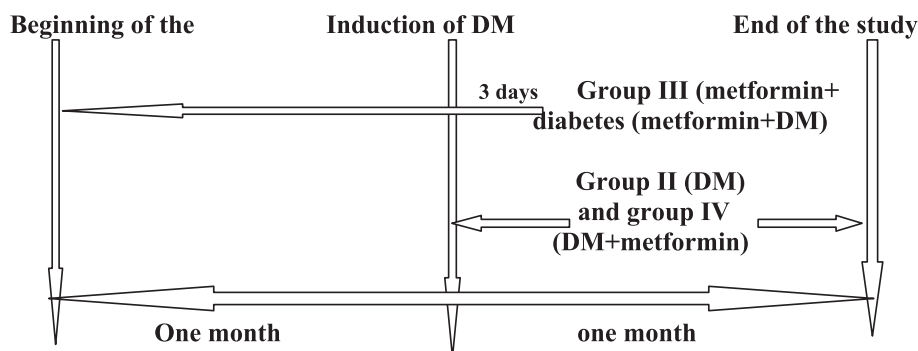
32 male albino rats, approximately aged 8 weeks and weighed 150–200 gram were housed in wire mesh cages at room temperature under ordinary living conditions. Rats were fed for whole period of study on standard laboratory rat diet (standard rat chow), Veterinary care was provided by laboratory animal house unit of faculty of Medicine, Cairo University.

Animals were randomly divided into the following 4 groups: *Group I: control group* ( $n = 8$ ): rats were injected citrate buffer and no further medications or life style modifications were applied.

*Group II: diabetic (DM) group* ( $n = 8$ ): rats were fasted for 12-h before induction of diabetes. Streptozotocin (STZ) was freshly dissolved in 0.05 M citrate buffer, pH 4.5 and injected intraperitoneally in a single dose of 40 mg/kg.<sup>4</sup> No further treatment or dietary modification was applied for 1 month.

*Group III: metformin + diabetes (metformin + DM) group* ( $n = 8$ ): rats administered 120 mg/kg metformin orally once daily<sup>9</sup> for 1 month before induction of diabetes and sacrificed after 3 days.

*Group IV: diabetes + metformin (DM + metformin) group* ( $n = 8$ ): rats were subjected for induction of diabetes followed by administration of 250 mg/kg metformin once daily (Foretz et al., 2010) for 1 month.



At the end of the study protocol, all animals were weighed in grams & their naso-anus lengths in cm while the rats were anesthetized with ether were measured to calculate their body mass index (**BMI**) as an index of obesity according to an equation formulated by<sup>17</sup>:

$$\text{BMI} = \text{Cubic root of weight in grams} \\ \times 1000/\text{Naso-Anal length in cms}$$

Subsequently, fasting blood samples were withdrawn retro-orbital using a capillary tube in for assessment of fasting serum glucose, fasting serum insulin, (HOMA-IR), serum triglycerides (TG) and serum high density lipoprotein (HDL) levels. Animals were then scarified followed by rapid excision of liver and pancreas for further assessment of SIRT1 level and activity and pancreatic DNA ladder.

Serum glucose level was tested by kits supplied by “Diamond Diagnostics”.<sup>53</sup> The serum insulin concentrations were measured by enzyme immunoassay using the rat insulin ELISA kits,<sup>22</sup> and HOMA-IR index was calculated as the product of fasting serum insulin ( $\mu\text{U/L}$ ) and fasting serum glucose ( $\text{mmol/L}$ ) divided by 22.5. HOMA more than 4.0 is diagnostic of insulin resistance.<sup>19</sup>

The serum triglyceride was measured by quantitative – enzymatic –colorimetric determination of triglycerides in serum.<sup>5</sup> However, HDL-Cholesterol is obtained through selective precipitation of LDL and VLDL lipoproteins, thus HDL lipoproteins remain in solution. HDL-Cholesterol in supernatant was treated as a sample for cholesterol assay, and after many reactions a colored compound was yielded. The color was measured at 546 nm and was proportional to HDL-Cholesterol concentration in sample when used as directed.<sup>41</sup>

Abcam’s SIRT1 Activity Assay Kit (Fluorometric) (ab156065) measures the activity of SIRT1 by the basic principle of changing a SIRT1 reaction into the activity of the protease. In order to measure the enzyme activity of SIRT1, which is the NAD dependent histone deacetylase, and its homolog, this kit is designed so that the activity of NAD dependent histone deacetylase can be measured under existence of Trichostatin A, which is the powerful inhibitor of HDACs.<sup>58</sup>

## 2.2. Detection of SIRT1 gene expression by real time-polymerase chain reaction (real time-PCR) in liver and pancreas<sup>43</sup>

### 2.2.1. RNA isolation and reverse transcription

RNA was extracted from the hepatic and pancreatic tissue homogenate using the RNeasy plus mini kit (Qiagen, Venlo, The Netherlands), according to the manufacturer’s instructions. Genomic DNA was eliminated by a DNase-on-column treatment supplied with the kit. The RNA concentration was determined spectrophotometrically at 260 nm using the NanoDrop ND-1000 spectrophotometer (ThermoFisher scientific, Waltham, USA) and RNA purity was checked by means of the absorbance ratio at 260/280 nm. RNA integrity was assessed by electrophoresis on 2% agarose gels. (1  $\mu\text{g}$ ) of RNA were used in the subsequent cDNA synthesis reaction, which was performed using the Reverse Transcription System (Promega, Leiden, The Netherlands). Total RNA was incubated at 70 °C for 10 min to prevent secondary structures. The RNA was supplemented with  $\text{MgCl}_2$  (25 mM), RTase buffer (10X), dNTP mixture (10 mM), oligod(t) primers, RNase inhibitor (20 U) and AMV reverse transcriptase (20 U/ $\mu\text{l}$ ). This mixture was incubated at 42 °C for 1 h.

### 2.2.2. Quantitative real time PCR

qPCR was performed in an optical 96-well plate with an ABI PRISM 7500 fast sequence detection system (Applied Biosystems, Carlsbad, California) and universal cycling conditions min 95 °C,

**Table 1**  
Primer sequences used for RT-PCR.

Primer	Sequence
Sirt1	Forward primer: 5'-.....-3' Reverse primer: 5'-.....-3'
GAPDH	forward: 5'-CTCCATTCTTCCACCTTTC-3' reverse: 5'-CTTGCTCTCAGTATCCTTGC-3'

40 cycles of 15 s at 95 °C and 60 s at 60 °C). Each 10  $\mu\text{l}$  reaction contained 5  $\mu\text{l}$

SYBR Green Master Mix (Applied Biosystems), 0.3  $\mu\text{l}$  gene-specific forward and reverse primers (10  $\mu\text{M}$ ), 2.5  $\mu\text{l}$  cDNA and 1.9  $\mu\text{l}$  nuclease-free water. The sequences of PCR primer pairs used for each gene are shown in Table 1. Data were analyzed with the ABI Prism sequence detection system software and quantified using the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the GAPDH genes R1.<sup>28</sup>

## 2.3. Detection of DNA fragmentation<sup>11</sup>

DNA was extracted from the pancreas using the kit supplied by Qiagen according to the manufacturer’s instructions. Then Gel electrophoresis was done for extracted DNA.

### 2.3.1. Statistical analysis

Data were coded and entered using the statistical package SPSS version 15. Data were summarized using mean, standard deviation and % change for the quantitative variables. Comparisons between groups were done using analysis of variance (ANOVA) and multiple comparisons (Post Hoc test) for the quantitative variables. P-values less than 0.05 were considered as statistically significant.<sup>10</sup>

## 3. Results

### 3.1. Comparison between BMI%, serum glucose (mmol/L), serum insulin ( $\mu\text{U/L}$ ) levels and HOMA in all studied groups

As observed in Table 2, BMI% was insignificantly increased by 6.47% ( $P = 0.290$ ) while fasting serum glucose ( $\text{mmol/L}$ ), serum insulin ( $\mu\text{U/L}$ ) and (HOMA) levels were significantly increased ( $P = 0.000$ ) by 163.04%, 79.16% and 378.70% respectively in diabetic group (GpII) compared to control group (GpI). Mean values in diabetic group were respectively 280.06  $\pm$  19.8; 12.81  $\pm$  1.94; 17.88  $\pm$  1.23 and 10.34  $\pm$  2.17 versus 263.05  $\pm$  9.68; 4.87  $\pm$  0.18; 9.98  $\pm$  1.24 and 2.16  $\pm$  0.24 in control group.

As shown in Table 2, administration of metformin for 1 month before induction of diabetes was associated with significant improvement in BMI% ( $P = 0.001$ ), serum glucose level, serum insulin level and HOMA ( $P = 0.000$ ) in which these parameters were lower than diabetic group (GpII) by 17.35%, 50.04%, 27.74% and 63.73% respectively. Mean values of BMI%, glucose, insulin and HOMA were 249.92  $\pm$  14.64, 6.40  $\pm$  1.15, 12.92  $\pm$  2.04 and 3.75  $\pm$  1.26 in GpIII versus 280.06  $\pm$  19.8, 12.81  $\pm$  1.94, 17.88  $\pm$  1.23 and 10.34  $\pm$  2.17 in GpII. Interestingly, compared to control values, BMI%, serum glucose level and HOMA in GpIII showed a non-significant higher difference ( $P > 0.05$ ) of 4.99%, 31.42% and 73.61% respectively while insulin was significantly higher by 29.46% ( $P = 0.008$ ). Mean values of BMI%, glucose, insulin and HOMA were respectively 249.92  $\pm$  14.64, 6.40  $\pm$  1.15, 12.92  $\pm$  2.04 and 3.75  $\pm$  1.26 in GpIII versus 263.05  $\pm$  9.68; 4.87  $\pm$  0.18; 9.98  $\pm$  1.24 and 2.16  $\pm$  0.24 in GpI.

**Table 2**  
Changes of BMI%, fasting serum glucose (mmol/L), fasting serum insulin ( $\mu$ U/L) levels and HOMA in all studied groups.

Parameter	GpI (control)	GpII (DM)	GpIII (metformin + DM)	GpIV (DM + metformin)
BMI%	263.05 $\pm$ 9.68	280.06 $\pm$ 19.8	249.92 <sup>*</sup> $\pm$ 14.64	267.95 $\pm$ 10.31
Glucose (mmol/L)	4.87 $\pm$ 0.18	12.81 $\pm$ 1.94	6.40 <sup>*</sup> $\pm$ 1.15	6.77 <sup>*</sup> $\pm$ 1.53
Insulin( $\mu$ U/L)	9.98 $\pm$ 1.24	17.88 $\pm$ 1.23	12.92 <sup>*</sup> $\pm$ 2.04	13.11 <sup>*</sup> $\pm$ 1.78
HOMA	2.16 $\pm$ 0.24	10.34 $\pm$ 2.17	3.75 <sup>*</sup> $\pm$ 1.26	4.04 <sup>*</sup> $\pm$ 1.52

Values are represented as mean  $\pm$  SD.

N.B: control group results have been reused from our previous publication.<sup>18</sup>

<sup>\*</sup> Significant change comparing corresponding values to GpII.

<sup>\*</sup> Significant change comparing corresponding values to GpI.

Also **Table 2** showed that administration of metformin for 1 month after inducing diabetes in GpIV had no significant effect ( $P > 0.05$ ) on BMI% relative to both diabetic and control groups. Mean values were 267.95  $\pm$  10.31 in GpIV versus 280.06  $\pm$  19.8 in GpII and 263.05  $\pm$  9.68 in GpI. However, a significant improvement ( $P = 0.000$ ) regarding fasting serum glucose (mmol/L), fasting serum insulin ( $\mu$ U/L) and HOMA were observed in GpIV compared to GpII. They were significantly decreased by 47.15%; 26.67% and 60.93% respectively in GpIV compared to GpII.

On the other hand, relative to GpI, fasting serum glucose and HOMA in GpIII showed insignificant difference ( $P > 0.05$ ); while serum insulin ( $\mu$ U/L) was significantly higher by 31.36% ( $P = 0.004$ ) in GpIII. Mean values for serum glucose, insulin and HOMA were respectively 6.77  $\pm$  1.53; 13.11  $\pm$  1.78; 4.04  $\pm$  1.52 in GpIV, versus 12.81  $\pm$  1.94; 17.88  $\pm$  1.23; 10.34  $\pm$  2.17 in GpII and 4.87  $\pm$  0.18; 9.98  $\pm$  1.24; 2.16  $\pm$  0.24 in GpI.

Our study showed that although the administration of metformin before induction of diabetes had a higher improving effect on fasting serum glucose, insulin and HOMA levels than post-diabetic metformin, yet this difference was insignificant ( $P > 0.05$ ).

Mean values were respectively 6.77  $\pm$  1.53; 13.11  $\pm$  1.78; 4.04  $\pm$  1.52 in GpIV; 6.40  $\pm$  1.15; 12.92  $\pm$  2.04; 3.75  $\pm$  1.26 in GpIII versus 12.81  $\pm$  1.94, 17.88  $\pm$  1.23; 10.34  $\pm$  2.17 in GpII as shown in **Table 2**. Regarding BMI%, it revealed no significant change in ( $P > 0.05$ ) comparing corresponding mean values in GpIV (267.95  $\pm$  10.31) versus GpIII (249.92  $\pm$  14.64).

### 3.2. Comparison between the serum TG (mg/dl) and HDL (mg/dl) levels in all studied groups

As shown in **Table 3**, serum TG level (mg/dl) was significantly increased by 68.56% ( $P = 0.000$ ), while serum HDL level (mg/dl) was significantly decreased by 37.2% ( $P = 0.000$ ) in diabetic group compared to control group. Mean values were 102.18  $\pm$  12.48; 31.72  $\pm$  1.65 in GpII versus 60.62  $\pm$  12.54; 50.52  $\pm$  4.08 respectively in GpI.

Similar to its beneficial effect on BMI%, serum glucose, insulin and HOMA, the pre-diabetic metformin lowered significantly the serum TG (mg/dl) level by 22.70% ( $P = 0.004$ ) and increased significantly the serum HDL (mg/dl) level by 24.53% ( $P = 0.041$ ). Mean values attained 78.99  $\pm$  8.81 and 39.50  $\pm$  5.07 respectively in GpIII versus 102.18  $\pm$  12.48 and 31.72  $\pm$  1.65 respectively in GpII.

**Table 3**  
Changes of TG (mg/dl) and HDL (mg/dl) levels in all studied groups.

Parameter	Gp I (control)	Gp II (DM)	GpIII (metformin + DM)	GpIV (DM + metformin)
TG (mg/dl)	60.62 $\pm$ 12.54	102.18 $\pm$ 12.48	78.99 <sup>*</sup> $\pm$ 8.81	59.30 <sup>∞</sup> $\pm$ 12.30
HDL (mg/dl)	50.52 $\pm$ 4.08	31.72 $\pm$ 1.65	39.50 <sup>*</sup> $\pm$ 5.07	44.38 <sup>∞</sup> $\pm$ 7.46

Values are represented as mean  $\pm$  SD.

<sup>\*</sup> Significant change comparing corresponding values to GpI.

<sup>\*</sup> Significant change comparing corresponding values to GpI.

<sup>∞</sup> Significant change comparing corresponding values in GpIV versus GpIII.

Despite this beneficial effect, full recovery in serum TG and HDL levels was not achieved. A significant difference by 30.30% ( $P = 0.042$ ) and  $-21.81\%$  ( $P = 0.001$ ) existed respectively comparing GpIII versus GpI. Mean values were 78.99  $\pm$  8.81; 39.50  $\pm$  5.07 in GpIII versus 60.62  $\pm$  12.54; 50.52  $\pm$  4.08 respectively in GpI (**Table 3**).

As observed in **Table 3**, a significant improvement ( $P = 0.000$ ) in serum TG by 41.97% and HDL levels (mg/dl) by 39.9% was recorded comparing corresponding values of post diabetic metformin administration in GpIV relative to GpII. Even more, compared to control group, no significant difference was noticed ( $P > 0.05$ ). Mean values of serum TG and HDL levels were respectively 59.30  $\pm$  12.30; 44.38  $\pm$  7.46 in GpIV versus 102.18  $\pm$  12.48; 31.72  $\pm$  1.65 in GpII and 60.62  $\pm$  12.54; 50.52  $\pm$  4.08 in GpI.

On the other hand, comparing the effect of pre-diabetic versus post-diabetic metformin, our results showed that TG level in GpIV was significantly ( $P = 0.022$ ) lower by 24.93% relative to GpIII. However, no significant difference ( $P > 0.05$ ) was recorded as regards serum HDL comparing both groups. Mean values were respectively 59.30  $\pm$  12.30; 44.38  $\pm$  7.46 in GpIV versus 78.99  $\pm$  8.81; 39.50  $\pm$  5.07 in GpIII (**Table 3**).

### 3.3. Comparison between the SIRT1 level and SIRT1 activity (ng/dl) in the liver and pancreas of all studied groups

As shown in **Table 4**, **Figs. 1 and 2**, SIRT1 level and activity in the liver were significantly ( $P = 0.000$ ) decreased by 54.84% and 55.81% respectively following induction of diabetes. Mean values were 0.98  $\pm$  0.22; 2.28  $\pm$  0.58 in GpII versus 2.17  $\pm$  0.35; 5.16  $\pm$  1.28 in GpI respectively.

We noticed that pre-diabetic metformin was significantly effective to counteract the reduction of SIRT1 level and activity in the liver and pancreas of diabetic group. Our results for GpIII showed a significant improvement in SIRT1 level and activity in liver (ng/dl) by 51.02% ( $P = 0.005$ ), 154.82% ( $P = 0.000$ ) respectively relative to GpII. Even more, no significant difference was detected comparing GpIII to control group ( $P > 0.05$ ). Mean values of SIRT1 level and activity in liver were respectively 1.48  $\pm$  0.41; 5.81  $\pm$  0.75 in GpIII versus 0.98  $\pm$  0.22; 2.28  $\pm$  0.58 in GpII and 2.17  $\pm$  0.35; 5.16  $\pm$  1.28 in GpI.

As shown in **Table 4**, **Figs. 3 and 4**, SIRT1 level and activity in pancreas were significantly decreased by 59.3% ( $P = 0.009$ ) and



**Table 4**

Changes of SIRT1 level (ng/dl) and SIRT1 activity (ng/dl) in liver and pancreas in all studied groups.

Parameter	GpI (control)	GpII (DM)	GpIII (metformin + DM)	GpIV (DM + metformin)
SIRT1 level (liver)	2.17 ± 0.35	0.98 ± 0.22	1.48 <sup>†</sup> ± 0.41	1.74 <sup>†*</sup> ± 0.33
SIRT1 activity (liver)	5.16 ± 1.28	2.28 ± 0.58	5.81 <sup>†</sup> ± 0.75	7.44 <sup>†*</sup> ± 1.12
SIRT1 level (pancreas)	1.72 ± 0.55	0.70 ± 0.25	1.94 <sup>†</sup> ± 0.57	1.90 <sup>†</sup> ± 0.87
SIRT1 activity (pancreas)	8.24 ± 1.13	2.99 ± 1.13	7.04 <sup>†</sup> ± 1.85	6.73 <sup>†</sup> ± 1.80

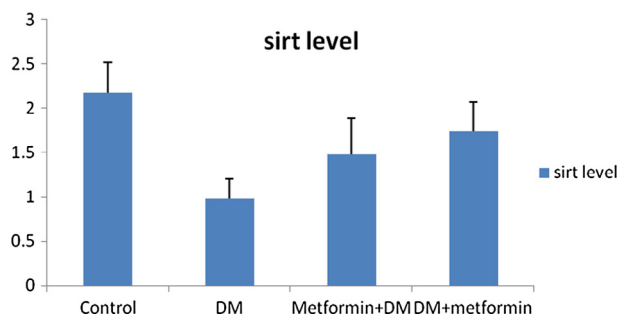
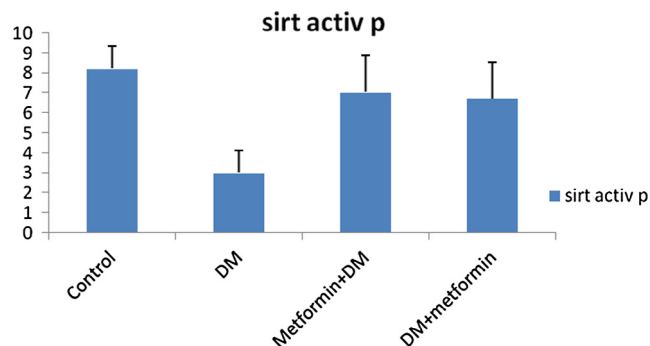
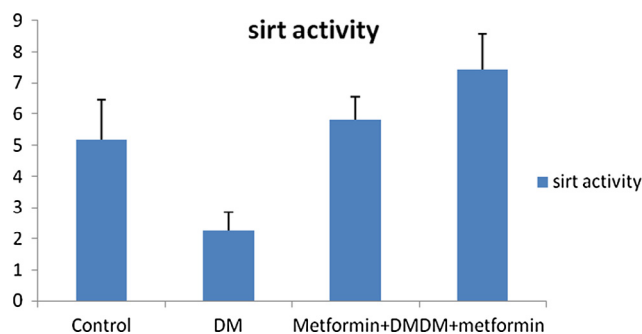
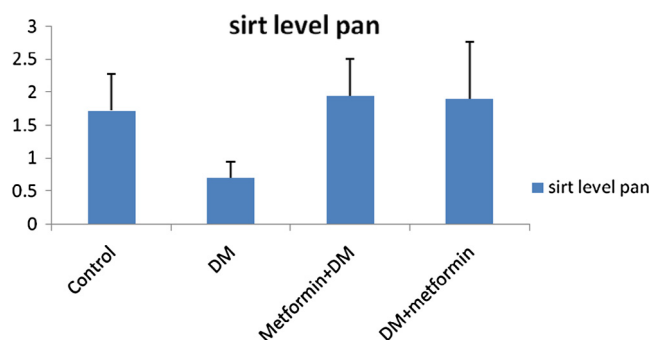
Values are represented as mean ± SD.

N.B: control group results have been reused from our previous publication.<sup>18</sup>

\*Significant change comparing corresponding values in GpIV versus GpIII

† Significant change comparing corresponding values to GpII.

• Significant change comparing corresponding values to GpI.

**Fig. 1.** Changes of SIRT1 level (ng/dl) in liver in all studied groups.**Fig. 4.** Changes of SIRT1 activity (ng/dl) in pancreas in all studied groups.**Fig. 2.** Changes of SIRT1 activity (ng/dl) in liver in all studied groups.**Fig. 3.** Changes of SIRT1 level (ng/dl) in pancreas in all studied groups.

63.71% ( $P = 0.000$ ) achieving mean values of  $0.70 \pm 0.25$ ;  $2.99 \pm 1.13$  in GpII versus  $1.72 \pm 0.55$ ;  $8.24 \pm 1.13$  in GpI respectively.

We also recorded significant elevation of SIRT1 level and activity in pancreas in GpIII by 177.14% ( $P = 0.001$ ) and 135.46% ( $P = 0.000$ ) relative to GpII and showed insignificant difference ( $P > 0.05$ ) compared to control group. Mean values were respec-

tively  $1.94 \pm 0.57$ ;  $7.4 \pm 1.85$  in GpIII versus  $0.70 \pm 0.25$ ;  $2.99 \pm 1.13$  in GpII and  $1.72 \pm 0.55$ ;  $8.24 \pm 1.13$  in GpI (Table 4).

Also, metformin intake for 1 month after induction of diabetes induced a significant enhancement in SIRT1 level and activity in the liver of diabetic rats. Data showed a significant increase ( $P = 0.000$ ) in SIRT1 level and activity in liver by 77.55% and 226.32% respectively in GpIV relative to diabetic group. Although relative to control group, SIRT1 level in liver of GpIV showed insignificant difference ( $P > 0.05$ ), yet a significant difference of 44.19% still existed ( $P = 0.003$ ) regarding the SIRT1 activity. Mean values were respectively  $1.74 \pm 0.33$ ;  $7.44 \pm 1.12$  in GpIV versus  $0.98 \pm 0.22$ ;  $2.28 \pm 0.58$  in GpII and  $2.17 \pm 0.35$ ;  $5.16 \pm 1.28$  in GpI (Table 4).

Similarly, the SIRT1 level and activity (ng/dl) in pancreatic tissues of GpIV exhibited significant elevation by 171.43% ( $P = 0.001$ ) and 125.08% ( $P = 0.000$ ) respectively compared to GpII. Moreover, no significant difference was detected relative to control group ( $P > 0.05$ ). Mean values were respectively  $1.90 \pm 0.87$ ;  $6.73 \pm 1.80$  in GpIV versus  $0.70 \pm 0.25$ ;  $2.99 \pm 1.13$  in GpII and  $1.72 \pm 0.55$ ;  $8.24 \pm 1.13$  in GpI (Table 4).

Interestingly, our results demonstrated that the difference in the improving effect of pre-diabetic metformin relative to post-diabetic metformin was insignificant ( $P > 0.05$ ). Mean values of SIRT1 level and activity in liver were respectively  $1.74 \pm 0.33$ ;  $7.44 \pm 1.12$  in GpIV versus  $1.48 \pm 0.41$ ;  $5.81 \pm 0.75$  in GpIII. As well, mean values of SIRT1 level and activity in pancreas were respectively  $1.90 \pm 0.87$ ;  $6.73 \pm 1.80$  in GpIV versus  $1.94 \pm 0.57$ ;  $7.04 \pm 1.85$  in GpIII (Table 4).

### 3.4. Changes in DNA ladder among all studied groups

The DNA fragmentation pattern was monitored in treated and untreated pancreatic tissue on agarose gel electrophoresis. Necrotic strand breaks/streaking DNA was observed diabetic group (GpII), but not in groups pretreated with metformin (GpIII) prior to STZ exposure and also in diabetic rats treated with metformin (GpIV) as revealed in Fig. 5.

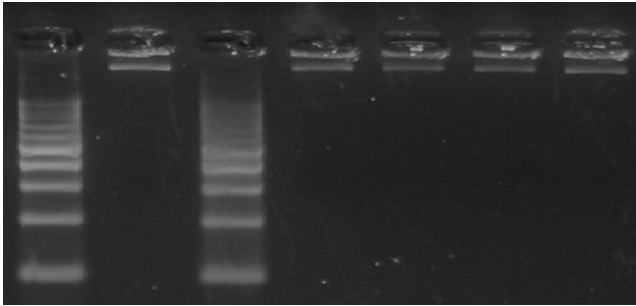


Fig. 5. DNA fragmentation pattern in pancreas among all studied groups.

Our results also showed that in diabetic group there was significant inverse relationship between (SIRT1 level and activity in liver and SIRT1 level in pancreas) and (BMI%, serum levels of glucose, insulin and TG, and HOMA) meanwhile the formers showed significant positive correlation with serum HDL level.

On the contrary, BMI%, serum levels of glucose, insulin, TG and HDL, and HOMA were not significantly correlated with the pancreatic SIRT1 activity.

However, regarding group III (metformin + DM) there was significant decrease in (BMI%, serum levels of insulin, TG and HDL, and HOMA) associated with (the elevation of SIRT1 level and activity in the liver and pancreas). Meanwhile the later showed significant positive correlation with serum HDL level. Although the serum glucose level was also significantly decreased parallel to the increase in SIRT1 activity in both liver and pancreas in rats received metformin before induction of diabetes yet its level was insignificantly correlated with SIRT1 level in liver and pancreas.

On the other hand, the elevated SIRT1 level and activity in hepatic and pancreatic tissues of diabetic rats following administration of metformin over 1 month was associated with significant diminution of BMI%, serum levels of glucose, insulin and TG, and HOMA) and significant increase in serum HDL level respectively .

#### 4. Discussion

The rising incidence of type 2 diabetes mellitus (T2DM) is a major public health problem in industrialized countries and new therapeutic strategies to treat and prevent T2DM are urgently needed worldwide.<sup>51</sup>

Abnormal elevation of hepatic gluconeogenesis is central to the onset of hyperglycaemia in patients with T2DM. Metformin corrects hyperglycaemia through inhibition of gluconeogenesis, but its mechanism of action is yet to be fully described.<sup>9</sup>

SIRT1 was originally identified as a NAD<sup>+</sup>-dependent histone deacetylase, has been identified as regulator of gluconeogenic gene expression.<sup>9</sup>. Growing evidence suggests that SIRT1 regulates glucose-lipid metabolism through its deacetylase activity for many known substrates. SIRT1 has also many roles in the metabolic pathways through its direct or indirect involvement in insulin signaling in insulin-sensitive organs, including adipose tissue, liver and skeletal muscles.<sup>24</sup> In addition, SIRT1 regulates insulin secretion, adiponectin production, inflammation, gluconeogenesis and oxidative stress, which together contribute to the development of insulin resistance.<sup>30</sup>

The present work has been designed to investigate the possible link of the action of metformin on SIRT1 in diabetic rats and if metformin could be a line of treatment that is helpful in re-establishing the physiological relevant activity of SIRT1 known to be attenuated with diabetes. We also attempted to test if it has any preventive value through tempering of SIRT1 disorders associated with the development of T2DM.

Therefore, we induced diabetes in eight adult male rats (GpII) by single intra-peritoneal injection of 40 mg/kg STZ, a well-known specific toxin causing pancreatic  $\beta$ -cell partial damage.<sup>4</sup> It was in agreement with our results, in which there was marked pancreatic apoptosis obtained after induction of diabetes.

Consequently, the fasting serum glucose level was significantly elevated, indicating hyperglycemia compared to control group. Moreover, our results also showed a significant increase in fasting serum insulin level and HOMA-IR confirming the characteristic features of T2DM. Results may be explained by the occurrence of insulin resistance in peripheral tissues especially skeletal muscles regarding their importance in glucose homeostasis.

Our results were in agreement with Kadowaki et al.<sup>26</sup> who stated that in streptozotocin (STZ)-induced diabetic rats, the number of insulin receptors is increased, whereas the receptor tyrosine kinase activity per unit of insulin binding is severely reduced. While, Burant et al.<sup>6</sup> suggested that peripheral insulin resistance associated with streptozotocin induced DM, may be related to modifications in insulin receptor structure and the glucose transport system, resulting in impaired signal transmission.

Beside, obtained data for BMI% showed a non-significant increase in diabetic group compared to control group. Our results could be primarily explained by the significant insulin resistance (hyperinsulinemia) which was in agreement with Takada et al.<sup>52</sup> who reported that diabetes associated polyphagia may be the cause of this non-significant weight change particularly that our rats in GpII were fed ad libitum and they may need a longer duration to exhibit weight loss.

Insulin resistance and T2DM are associated with a clustering of interrelated plasma lipid and lipoprotein abnormalities, which include reduced HDL cholesterol and elevated TG levels.<sup>8</sup> Similarly, our results showed significant elevation of serum TG level by 68.56% and significant reduction of serum HDL level by 37.2% in diabetic rats versus the control group.

As well, Krauss<sup>32</sup> emphasized that insulin resistance may play a pivotal role in the development of diabetic dyslipidemia by influencing several factors. Increased efflux of free fatty acids from adipose tissues and impaired insulin-mediated skeletal muscle uptake of free fatty acids, increase fatty acids flux to the liver.

Beside, insulin resistance also increases hepatic lipase activity which is responsible for hydrolysis of phospholipids in LDL and HDL particles and leads to smaller and denser LDL particles and a decrease in HDL.<sup>39</sup>

As Caton et al.<sup>9</sup> demonstrated that SIRT1 is implicated in the regulation of glucose metabolism and insulin sensitivity we thought that studying the associated disorders in SIRT1 might be beneficial. Our results showed that SIRT1 level and activity were decreased by 54.84% and 55.81% respectively in the liver and by 59.30% and 63.71% respectively in the pancreas of diabetic rats. Remarkably, there was marked pancreatic apoptosis after induction of diabetes.

Our results were in agreement with Suvarna<sup>50</sup> who found that the nutrients availability such as high serum glucose in diabetic rats are associated with decreased levels of NAD/NADH<sup>+</sup> which are the main activators of SIRT1- leading to decreased SIRT1 release and activity.

However, we could not specify if the decrease in SIRT1 shared partially in the development of hyperglycemia and IR or the STZ-induced T2DM was the initial cause of the observed decrease in SIRT1.

Meanwhile, our results in GpII revealed that the elevated serum glucose, insulin, HOMA and TG were parallel to the decline in SIRT1 level and activity (inverse correlation). Regarding the changes in serum HDL and SIRT1, both showed a positive correlation.

Data could be explained by Sebastián et al.<sup>49</sup> who suggested that a combination of over-nutrition, inactivity, genetic and other

factors interact to produce a state of metabolic susceptibility that leads to dysregulation of AMPK and SIRT1. This in turn could lead to insulin resistance, subsequent hyperinsulinemia, mitochondrial dysfunction, and abnormalities in cellular lipid metabolism.

Wang et al.<sup>54</sup> showed that hepatic-specific deletion of SIRT1, lead to hepatic glucose overproduction and hyperglycemia. The resulting increase in ROS impairs insulin signaling in insulin-sensitive tissues, such as adipose tissues and muscles, leading to insulin resistance.

In addition, Bordone et al.<sup>3</sup> showed that a reduction in SIRT1 levels reduces the capacity of  $\beta$ -cells to secrete insulin in response to glucose. This fact was explained by Rogina and Helfand<sup>47</sup> who noticed elevated levels of UCP2 in cells with reduced SIRT1. Thus ATP synthesis was reduced lowering the amplitude of insulin induction by glucose.

Metformin has been shown to be highly effective in delaying or preventing T2DM in those having IGT.<sup>13</sup> Parallel, in our study, rats administered metformin (120 mg/kg) once daily for a period of one month before induction of diabetes, showed significant decrease in their BMI%, serum glucose level, serum insulin level and HOMA when compared to diabetic group (GpII).

The diabetes prevention program (DPP) demonstrated that intervention with metformin decreased the development of diabetes in adults with impaired glucose tolerance by 31%.<sup>15</sup> Also, Mark et al.<sup>37</sup> were in agreement with our results who demonstrated that metformin administration to normal rats is capable of preventing the development of acute lipid-induced insulin resistance. The primary effect of metformin was amelioration of the lipid-impaired insulin suppressibility of HGO; thus, the liver would seem to be the principal target tissue for this prophylactic effect.

In the same context, Mithieux et al.<sup>38</sup> reported that in hyperinsulinemic-euglycemic clamping (HEC) conditions, metformin pre-treatment resulted in increased storage of liver glycogen, in high-fat-fed rats, suggesting that metformin attenuated glycogenolysis or enhanced glycogen synthesis, perhaps from gluconeogenic substrates.

On the other hand, Kim et al.<sup>29</sup> examined chronically insulin-resistant high-fat-fed rats under HEC conditions and demonstrated adverse effects on HGO before the manifestation of attenuated peripheral glucose disposal. There was no accompanying reduction in triacylglycerol storage in liver after acute lipid infusion and HEC suggesting that this is not the primary mechanism for the effect of metformin on HGO.

Scientists have implicated enhancement of liver signaling in the mechanism of metformin effect, as the drug induced increases in AMPK activity along with reduced JNK protein<sup>21</sup> and in skeletal muscle of individuals with T2DM after 4 weeks' administration of the drug.<sup>40</sup>

In contrast, no effect of metformin was detected on AMPK activity in red or white muscle or a central fat depot, consistent with a lack of effect of the drug on peripheral glucose disposal.<sup>37</sup> Therefore, Samuel et al.<sup>48</sup> suggested that the effects of metformin on gluconeogenesis may be in part mediated through its effects on other important gluconeogenic enzymes, such as pyruvate carboxylase, pyruvate kinase or glucose 6-phosphatase.

Overall, these findings may help in explaining the prophylactic effect of metformin to oppose further deterioration in glucose regulation in humans with impaired glucose tolerance or obesity<sup>31</sup> and in implicating enhanced liver signaling in the therapeutic mechanism of action of metformin.

Similar to its beneficial effects on BMI%, serum glucose, insulin and HOMA, when given for 1 month before induction of diabetes in GpIII, metformin was also found to significantly reduced the serum TG level and the serum HDL level was increased significantly relative to diabetic rats in GpII.

Our results were in agreement with Goldberg et al.<sup>23</sup> who reported that metformin modestly reduced small and dense LDL and raised small and large HDL. They suggested that the effect of metformin to increase small HDL was independent of adiponectin, BMI, and insulin resistance. These findings support the notion that metformin use may be considered as prophylactic therapy aimed at lowering cardiovascular risk factors.<sup>2</sup>

Furthermore, this study showed that metformin administration over 1 month before induction of diabetes was associated with less reduction in the level and activity of SIRT1 in liver than that occurred in diabetic rats. Additionally, no apoptotic changes were detected in the pancreas of GpIII.

Yoshizaki et al.<sup>57</sup> suggested that this cross talk between metformin and SIRT1 may be of additive role in protection against obesity, dyslipidemia, pancreatic inflammation, apoptosis and IR thus preventing or at least delaying the occurrence of T2DM.

Interestingly, our results revealed that the significant increase in SIRT1 was associated with significant increase in HDL level (+ve correlation) although it was negatively correlated with BMI %, serum levels of glucose, insulin, TG and HOMA.

However, it is not clear whether metformin improved hyperglycemia and hence spared SIRT1 from the negative feedback of the low levels of NAD<sup>+</sup>/NADH or metformin activated SIRT1 via AMPK signaling pathway which in turn improved diabetes mellitus. In support, there is a striking resemblance between the pleiotropic effects of metformin and the physiological features of SIRT1 activation.<sup>55</sup> In both cases reduced inflammation and oxidative stress, weight loss, improvement in plasma lipid profile as well as reduced the beta cells destruction (apoptosis) take place.<sup>56</sup>

Our study tried to evaluate which of the pre- or post-diabetic metformin was more beneficial? Undoubtedly, metformin has been in wide clinical use for the alleviation of the hyperglycemia associated with T2DM for decades.<sup>44</sup> Concomitant with this, our results showed that fasting serum glucose level (mmol/L), fasting serum insulin level ( $\mu$ IU/L) and HOMA were significantly decreased in GpIV compared to GpII. Meanwhile, BMI% was insignificantly decreased in GpIV compared to GpII.

Despite this, a comprehensive molecular basis for the antihyperglycemic mechanism of action of metformin has yet to be established. Whereas Kumar and Dey<sup>33</sup> reported that metformin was shown to ameliorate defects in insulin signaling in vitro, involving activation of IRSs and associated phosphatidylinositol 3 kinase activity.

In addition, metformin activates AMPK in hepatocytes,<sup>21</sup> whereas enzyme activity was elevated in skeletal muscle of diabetic patients after 4 weeks of metformin treatment.<sup>40</sup>

Metformin treatment also had positive effects on the insulin signaling pathway in the liver.<sup>36</sup> Metformin induces reductions in JNKs which have been shown to phosphorylate IRS-1 in vitro thus reducing its activity. This could be important in mediating the ability of metformin to reduce the impact of lipid infusion on insulin action in the liver.<sup>27</sup>

Our study revealed that the serum HDL level was significantly higher in GpIV (DM + metformin) relative to diabetic GpII. Moreover, the serum TG level in GpIV was significantly decreased relative to GpII.

Our results were in agreement with Afzal et al.<sup>1</sup> who observed a significant elevation in the level of TG and reduction in HDL in the animals exposed to intraperitoneal administration of chemical carcinogen diethyl nitrosamine, when compared to a normal control but these parameters were maintained to normal by the treatment of metformin. Also, Rautio et al.<sup>45</sup> reported that metformin was effective in improving several parameters of lipid profile in T2DM and non-diabetics with coronary heart disease.



Contrary to our results, Cibula et al.<sup>12</sup> demonstrated that in T2DM and non-diabetics with central fat distribution, metformin reduced cholesterol but not triglycerides. Fleming et al.<sup>20</sup> revealed that metformin use in women with PCOS have demonstrated an improvement of HDL but no significant effect on total cholesterol or triglycerides.

The divergence of the findings among these studies may be related to the characteristics of the population studied; thus, for example metformin may be more effective in more hyperinsulinemic and/or obese populations.

As observed in our work, metformin intake for 1 month after induction of diabetes induced a significant enhancement in SIRT1 level and activity in the liver and pancreas of diabetic rats relative to diabetic group (GpII). Our results showed significant elevation of SIRT1 level and activity in pancreas in GpIV relative to GpII. In addition the pancreatic apoptosis was disappeared.

Metformin has been reported to activate AMPK. Canto et al.<sup>7</sup> suggested that AMPK can increase SIRT1 activity potentially through an AMPK-mediated increase in the transcription of (NAMPT), the rate-limiting enzyme of the salvage pathway for NAD<sup>+</sup>, an essential co-factor for SIRT1 activity. Additionally, Hou et al.<sup>24</sup> have reported that SIRT1 can deacetylate and activate LKB1, leading to the activation of AMPK. This raises the possibility that a positive feedback system could be operative in response to hyperglycaemia following metformin administration.

Interestingly, Lee et al.<sup>34</sup> found that metformin-treated cells produced a lower amount of NO than control cells, which leads to resistance to cytokine toxicity. Authors also suggested that metformin through activation of SIRT1 plays a cytoprotective role against cytokine in pancreatic  $\beta$ -cells. SIRT1 might deacetylate non-histone proteins such as NF- $\kappa$ B protecting pancreatic islets from cytokine toxicity.

Finally, we should ask which was more beneficial: pre- or post-diabetic metformin? Unexpectedly, data in our work found no significant difference between the effect of metformin pre- or post-diabetic regarding BMI%, serum glucose, insulin, HDL, HOMA and pancreatic apoptosis, while serum TG level was significantly lower in GpIV.

Notably, when we compare the effect of metformin versus the effect of caloric restriction CR used in our previous publication,<sup>18</sup> we did not detect any significant difference between the effect of metformin or CR (either before or after) induction of diabetes on all the studied parameters which may be explained by Dhahbi et al.<sup>14</sup> who proposed that metformin is CR mimetic. They found that mice administered the drug exhibit similar gene expression changes as CR mice.

### Conflict of interest

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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