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An AMPK activator, Metformin, ameliorates aging-related oxidative hepatic injury induced by D-galactose *via* Irisin/ SIRT1 pathway, a possible role of PPAR-γ besides its antioxidant potency in rats.

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Keywords

- Aging
- Metformin
- Oxidative stress
- Irisin/SIRT1/PPAR-γ
- BADGE

Abstract

Background: While many studies have investigated the metabolic and cellular effect of the AMPK activator anti-diabetic drug metformin, we have not come across a paper that deals with the molecular mechanism of its anti-aging impact *via* irisin hormone in a SIRT1/ PPAR-γ-dependent manner. **Materials and Method:** For this purpose, we conducted, besides the control group, an experimentally induced aging model through D-galactose and compared its solo effect *versus* the combined metformin and D-galactose administration. Furthermore, another group pretreated by Bisphenol-A-diglycidyl ether (BADGE), a selective PPAR-γ antagonist, was conducted. **Results:** Metformin blunted aging-associated hepatocellular changes in both structural and functional aspects as revealed by biochemical and histopathological evaluations. Also, it maintained glucose homeostasis. Such effects were accompanied bypreserving redox homeostasis and boosting the level ofIrisin andSIRT1proteins. A mechanism that was, in part, dependent on PPAR-γ as evidenced after its blockade by BADGE. There was an observable negative correlation between serum irisin and ALT, HOMA-IR, and MDA while a positive correlation with total antioxidant capacity (TAC). **Conclusion:** The results came up with the geroprotective impact of metformin and its benefits could extend far beyond diabetes. It appears to be a promising anti-aging pill. It could retard aging-associated hepatic alterations and/or its bad consequences progression.

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Introduction

Aging is a natural life stage with a reduced adaptation and the inability to keep homeostasis. It is associated with organ structural and functional changes that make the body more susceptible to stress and damage [1].Worldwide, the geriatric population is growing with an increase in disability and illness [2]. Considering the liver, aging is associated with several changes, such as insulin resistance, decreased hepatic flow, and hepatocytes and endothelial cells damage especially in vulnerable elderly patients [3]. Therefore, recognizing the pathophysiology of hepatic aging is a significant argument for medical gerontology to promote healthy aging.

Currently, an increasing number of evidence indicate that chronic D-galactose (D-gal) administration produces a valuable aging model in rats, with biochemical and histopathological changes that mimic the morphological and functional characteristics of the naturally aged liver [3]. D-gal is a naturally occurring aldohexose in the body. However, at high concentrations, it can be converted into aldose and hydroperoxide with the formation of reactive oxygen species (ROS). Furthermore, D-gal interacts with free amine *via* non-enzymatic glycation, resulting in the formation of advanced glycation products with another series of ROS. On a ROS generation basis, oxidative damage is crucial in the pathophysiology of hepatic aging. When the endogenous antioxidants are exhausted, free radical scavenging becomes insufficient with the start of negative consequences [4]. Therefore, improving the antioxidant potency may be valuable to slow the aging process.

In aging studies, Silent information regulator 1, sirtuin 1, (SIRT1) becomes an attractive target. It modulates transcriptional factors associated with increasing life expectancy. It is a nicotinamide adenine dinucleotide (NAD) deacetylase that is normally expressed in hepatic tissue and is a key player in its physiology. It is involved in senescence, oxidative injury, glucose homeostasis, and the cell cycle. It is down-regulated in aging-related hepatic fibrosis. Likewise, the reduced hepatic SIRT1 could aggravate aging primarily *via* decreasing peroxisome proliferator-activated receptor-γ (PPAR-γ), which is a nuclear receptor expressed in the liver [5]. Furthermore, irisin is a newly discovered hormone that modulates metabolic processes, maintains glucose homeostasis, improves hepatic insulin resistance, and reduces aging-related pathologies. Recently, irisin down-regulation was linked to the aging process [6, 7]. Therefore, a rise in irisin and targeting SIRT1 / PPAR-γ could causally promote healthy aging.

Scientists have made remarkable progress in extending health span and longevity through pharmacological approaches over the last few decades. One of these is Metformin (Met). It is an AMPK activator agent that is taken orally to control the glycemic state in patients with non-insulin-dependent diabetes mellitus (NIDDM) [2]. Recently, Met is tested clinically for its age-targeting effects and named TAME (targeting aging by Met). Furthermore, it is considered a safe, cheap, and abundant drug with numerous molecular mechanisms such as anti-apoptotic, mitochondrial function improvement, and autophagy induction in addition to a long history of clinical use with decades-old safety. These

make Met an outstanding candidate in aging research [8].However, to our knowledge, there are no reports to date about the ameliorative effects of Met in hepatic aging *via* irisin hormone in a SIRT1/PPAR-γ-dependent manner. Therefore, in view of these considerations, the current study was carried out to evaluate the protective properties of Met against D-gal-induced hepatic aging and to elucidate the potential involvement of the SIRT1/ PPAR-γ/ irisin pathway in such impact.

2. Materials and methods

2.1. Animals used:

The present research was carried out on thirty Wistar albino male rats, 6-8 weeks old, weighing 200±20 g, and obtained from the Experimental Animal Unit of the Faculty of Agriculture, Benha University, Egypt. Prior to the initiation of the experiment, the rats were acclimated for 10 days (temperature 25 °C ± 1°C) and were given a standard laboratory commercial diet and water ad libitum. The experimental procedures, animal handling, sampling, and scarification were done in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition (2011).Ethical approval from the Ethics Committee of the Faculty of Medicine, Benha University, for the study protocol and utilization of rats, was obtained (Approval no MD11072020).

2.2. Drugs

D- galactose, purity > 99%, and Dimethyle sulfoxide, (DMSO) were purchased from LOBA chemie (Mumbai, India). Metformin (Glucophage, 1000 mg) was obtained from Merck/Mina Pharm (Cairo, Egypt). Bisphenol A diglycidyle ether (BADGE) was purchased from Sigma Chemical (St. Louis, MO, USA).

2.3. Experimental groups and procedure:

The rats were assigned into five groups: (*n=6*) each. Group 1 (vehicle control); rats were given equivalent volumes of saline and DMSO solvents intra-peritoneal (i.p) and distilled water by oral gavage. Group 2 (D-gal); rats were injected with D-gal i.p dissolved in saline at a dose of 300 mg/kg/day/5 days/week for 6 weeks [9]. Group 3 (Met); rats were given Met (300 mg/kg/daily) dissolved in distilled water by oral gavage for 6 weeks [10]. Group 4 (Met + D-gal); rats were administrated both Met (300 mg/kg/daily, oral gavage), and D-gal (300 mg/kg/day/5 days/week, *i.p)*, for 6 weeks. In group 5 (BADGE + Met + D-gal) the rats were treated with BADGE dissolved in DMSO (15 mg/kg, daily, *i.p*), 24-hsbefore Met (300 mg/kg, daily, oral gavage)and D-gal (300 mg/kg/day/5 days/week, *i.p)* administration for 6 weeks [11].

2.4. Sampling

At the end of the experiment, after overnight fasting, the animals were anesthetized with Sodium pentobarbital (40 mg/kg).Then a midline incision was made. Blood samples were obtained from the abdominal aorta and left at room temperature for serum isolation. The obtained serum was kept at -20 ^{o} C. Finally, the liver was harvested and divided into 2 parts; apart was immersed in ice-cold phosphate-buffered saline (PBS) for estimation of oxidative stress-related markers, PPAR-γ, and irisin protein levels, while the other part was kept in formalin for histopathology and immune-histochemical expression of SIRT1 expression [12]. An illustration of the experimental procedure was shown in Fig.1.

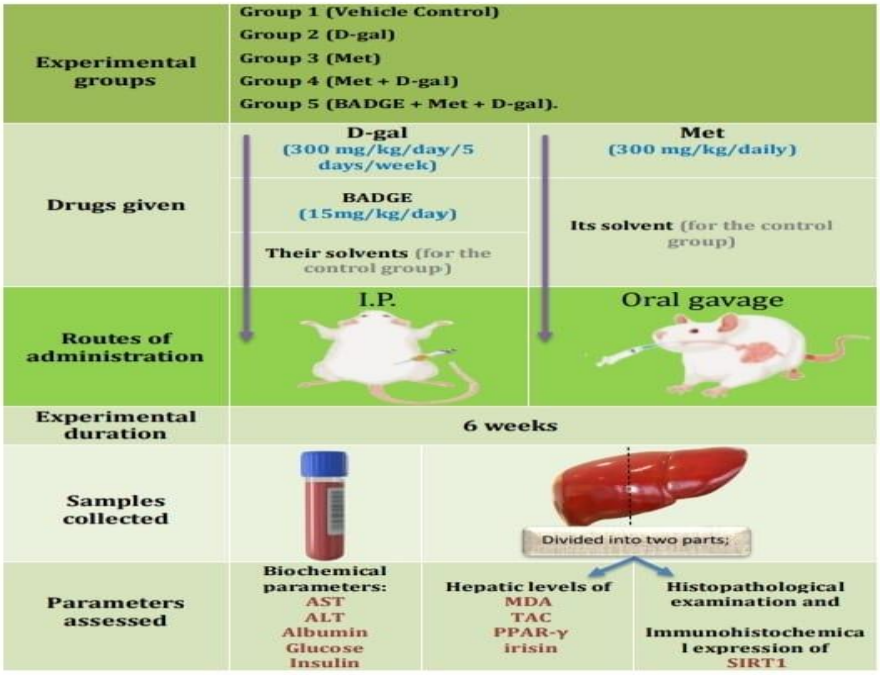

Fig 1: Illustration of the experimental procedure

D-gal; d-galactose, Met; metformin, BADGE; Bisphenol A diglycidyl ether, I.P.; Intra-peritoneal injection, AST; Aspartate aminotransferase, ALT; Alanine aminotransferase, MDA; malondialdehyde, TAC; Total antioxidant capacity, PPAR-γ; Peroxisome proliferator-activated receptor gamma, SIRT1; Sirtuin 1, or Silent information regulator 1.

2.5. Serum biochemical assay of hepatic functions-related parameters

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) [13], and albumin [14] were assessed using automated biochemical analyzer (BT-1500, Biotecnica instruments, Italy), by the commercially available kits obtained from (Bio diagnostics Co, Giza, Egypt)

2.6. Serum assay of glucose homeostasis-related parameters

Serum glucose was measured using diagnostic kits obtained from (catalog No: GL 13 20, Biodiagnostics Co, Cairo, Egypt) by enzymatic colorimetric method [15], while serum insulin was assessed using ELISA kits (catalog No: RA19004R, BioVendo Brno, Czech), with the resulting antibody-antigen complex assessed at 415 nm in a Stat fax 2600 plate reader [16].

Insulin resistance (IR) was measured by the homeostasis model assessment of insulin resistance (HOMA-IR), a well-documented marker for IR, that was calculated according to the following formula [17]: HOMA-IR = (fasting glucose (mg/dl) × fasting insulin (μU/mL) / 405).

2.7. Measurement of hepatic lipid peroxidation and total antioxidant capacity

The hepatic tissue samples were separated and washed in phosphate-buffered saline (PBS) containing 0.16 mg/mL heparin to segregate RBCs and serum clots. For each 1 g of tissue, 5 mL buffer (50 mM potassium phosphate, pH 7.5, 1 mM EDTA) was homogenized using a sonicator homogenizer. Aliquots of tissue homogenate were centrifuged at 4000 revolutions per minute (rpm) for 20 minutes in a refrigerated centrifuge and stored at - 80 ^{o}C until use.

The malondialdehyde (MDA) level (catalog No: MD 25 29), and total antioxidant capacity (TAC) level(catalog No: TA 25 13) were used to assess the oxidative status using kits obtained from Biodiagnostic Company, Cairo, Egypt as previously described by Uchiyama and Mihara [18] and Prior and Cao [19] respectively.

2.8. Evaluation of hepatic protein levels of PPAR-γ and irisin by ELISA

PPAR-γ was assessed using rats ELISA kits obtained from (catalog No: abx155996, Abbexa, LLC, USA) [20]. Irisin was quantified using rats' ELISA kits (catalog No: MBS 260144, MyBiosource, Germany) [21].

2.9. Histopathological evaluation of hepatic tissue

The preserved hepatic tissue in 10 % formalin was processed using conventional paraffin-embedding techniques. The paraffin blocks were cut into sections (5 µm thickness), mounted on glass microscope slides, deparaffinized, and then stained with Hematoxylin & Eosin for light microscopic inspection [22]. The hepatic histopathological injury was scored [23-25]. The scoring criteria are shown in Table 1.

Table 1: Scoring criteria for hepatic histopathological injury

Criteria	Score			
	0	1	2	3
Vascular state	----	Dilated vein	Dilated congested vein	Dilated congested veins and sinusoids
Number of inflammatory foci/200x field	----	< 2	2 – 4	> 4
Necrotic hepatocytes %	----	5 - 10	> 10	> 25
Fatty changes %	----	5 - 33	33 – 66	> 66

2.10. Assessment of Immunohistochemical expression of hepatic SIRT1 protein level

Tissue sections were embedded in paraffin and dehydrated using a gradated alcohol series for immunohistochemical analysis. For antigen retrieval, 0.01 M sodium citrate buffer was heated to 80 C for 12 minutes. After that, the sections were incubated for 24 h with an anti-SIRT1 (Abcam Cambridge, UK) provided kits primary monoclonal antibody. An avidin-biotin complex (ABC kit, Vector Laboratories) was used to visualize the staining, which was incubated for one h at 37 C. The final visualization was achieved through a reaction with 3,3-diaminobenzidine tetrahydrochloride and the use of a counterstain (Mayer's hematoxylin). The immunohistochemistry data were scored as follows: 0 for no staining, 1 for mild staining, 2 for moderate staining, and 3 for strong staining [26].

2.11. Statistical analysis:

SPSS (Version 20) was used for statistical analysis (SPSS Inc., Chicago, USA). One-way ANOVA was used to estimate group differences, and the Least Significant Difference (LSD) test was used as a post hoc test. Kruskal Wallis test was used for the qualitative variables. All values were expressed as mean ± SD. Pearson's correlation coefficient (r) 2-tailed test was used to examine the relationships between irisin and ALT, HOMA-IR, TAC, and MDA levels. A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Effect of D-gal and Met administration on serum AST, ALT, and albumin in the experimental groups

The data presented in Table 2 show that D-gal has deleterious effects on the liver that mimic those of natural aging. It was evidenced by an observable increase in serum levels of hepatic enzymes AST and ALT, but a decline in serum albumin when compared to the control group ($P < 0.05$). On the contrary, Met and D-gal co-treatment significantly preserved hepatic functions and reversed these parameters when compared to D-gal-treated rats. This protection was substantially declined on administrating BADGE prior to Met + D-gal ($P < 0.05$).

Table 2: Serum biochemical parameters related to hepatic function

Parameters	Experimental groups				
	Control	D- gal	Met	Met + D- gal	BADGE + Met + D-gal
AST (U/L)	93.46 ± 5.75	163.54 ±5.34[a]	94.69 ± 4.18[b]	115.31±3.19[a,b,c]	157.31±6.15 [a,b,c,d]
ALT(U/L)	33.61 ± 1.51	59.97 ± 2.41[a]	32.97 ± 2.05[b]	39.57 ±0.86 [a,b,c]	57.33±1.61 [a,b,c,d]
Albumin (gm/dl)	3.34 ± 0.23	2.77 ± 0.14 [a]	3.35± 0.07 [b]	3.34 ± 0.12 [b]	2.95 ± 0.09 [a,b,c,d]

Data are expressed as mean ± standard deviation SD; $n = 6$, [a] $P < 0.05$ significant difference compared with the control group; [b] $P < 0.05$ significant difference compared with the D-gal group; [c] $P < 0.05$ significant difference compared with the Met group; [d] $P < 0.05$ significant difference compared with the Met + D-gal group. D-gal; d-galactose, Met; metformin, BADGE; Bisphenol A diglycidyl ether, AST; Aspartate aminotransferase, ALT; Alanine aminotransferase.

3.2. Effect of D-gal and Met administration on serum glucose, insulin, and HOMA-IR in the experimental groups

The data in Table 3 show that D-gal induced glucose dyshomeostasis and significant insulin resistance; D-gal-treated rats exhibited an observable increase in the serum glucose, insulin, and calculated HOMA-IR ($P < 0.05$). Furthermore, Met co-treatment significantly prevented such an increase when compared to D-gal. This protection was substantially declined on administrating BADGE prior to Met + D-gal ($P < 0.05$).

Table 3: Glucose homeostasis-related parameters

Parameters	Experimental groups				
	Control	D- gal	Met	Met + D- gal	BADGE + Met + D-gal
Glucose (mg/dl)	87.7 ± 4.31	262.2 ± 44.85[a]	82.93 ± 3.02[b]	95.45 ±4.51[b]	198.43±11.17[a,b,c,d]
Insulin (μU/mL)	3.22 ± 0.66	11.82 ± 1.75 [a]	2.94 ± 0.68 [b]	3.57 ± 0.5 [b]	8.27 ± 0.78 [a,b,c,d]
HOMA-IR	0.7 ± 0.17	7.68 ± 2.01[a]	0.6 ± 0.16 [b]	0.83 ± 0.13 [b]	4.44 ± 0.37 [a,b,c,d]

Data are expressed as mean ± standard deviation SD; $n = 6$, [a] $P < 0.05$ significant difference compared with the control group; [b] $P < 0.05$ significant difference compared with the D-gal group; [c] $P < 0.05$ significant difference compared with the Met group; [d] $P < 0.05$ significant difference compared with the Met + D-gal group. D-gal; d-galactose, Met; metformin, BADGE; Bisphenol A diglycidyl ether, HOMA-IR; Homeostatic model assessment for insulin resistance.

3.3 Effect of D-gal and Met administration on hepatic histopathology in the experimental groups (Fig. 2)

To ensure hepatic affection at the structural levels, a histopathological evaluation was done. The control and Met groups showed a normal thread-like arrangement of hepatocytes with normal central veins (Fig. 2A and 2C respectively). On the other hand, D-gal treatment caused marked hepatic cells disturbances, necrosis, fatty changes, inflammatory cell infiltration, and vascular congestion (Fig. 2B). Noteworthy, combined administrations of Met + D-gal preserved the hepatic architecture with minimal peri-portal inflammatory cell infiltration (Fig. 2D). Moreover, the BADGE treatment with both Met + D-gal group partially inhibited the hepato-protective effect of Met against the D-gal induced changes. This group showed notably some inflammatory cell infiltrate, some dilated congested veins, and some fatty changes in the hepatocyte (Fig. 2E).

Also in this regard, there was a significant increase in the hepatic injury score in the D-gal group versus the control while an observable decline in the Met +D-gal treated group versus the D-gal group ($P < 0.05$). Such improvement in the hepatic injury score was further attenuated by treatment with BADGE (Fig. 2F).

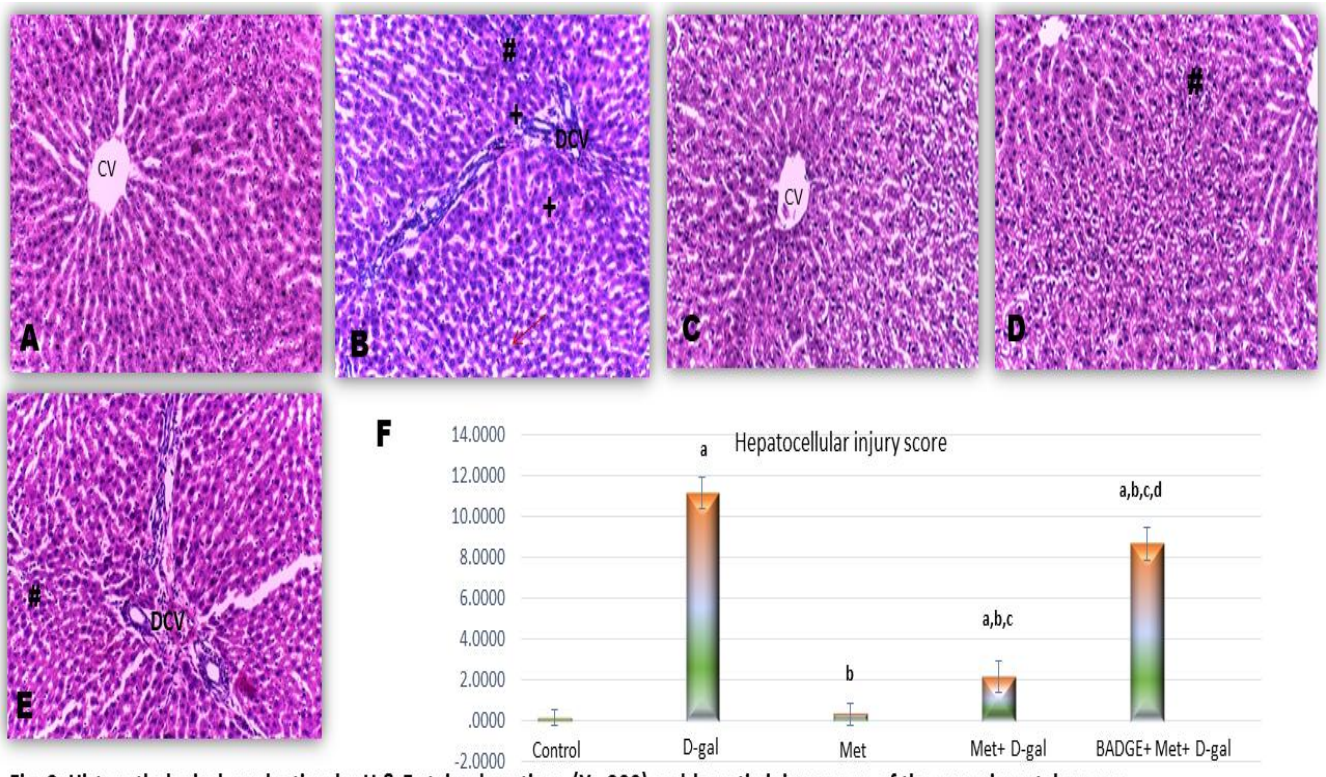

Fig. 2: Histopathological evaluation by H & E-stained sections (X= 200) and hepatic injury score of the experimental groups

2-A): The control group shows a normal hepatic architecture with a thread-like arrangement of hepatocytes with a normal central vein (**CV**). **(2-B):** D-gal group exhibits marked hepatic cell disruptions; inflammatory cell infiltration (#), necrotic (+), fatty changes (→), and vascular congestion and sinusoidal dilatation (DCV). **(2-C):** Met group shows normal liver histology. **(2-D):** Met + D-gal group demonstrates remarkable improvement in liver histology with minimal inflammatory cell infiltrations (#). **(2-E):** BADGE + Met + D-gal group shows dilated congested vein (DCV) and moderate inflammatory cell infiltrate. **(2-F):** Hepatic injury score of the experimental groups, data are expressed as mean ± standard deviation SD; $n = 6$, $^{a}P < 0.05$ significant difference compared with the control group; $^{b}P < 0.05$ significant difference compared with the D-gal group; $^{c}P < 0.05$ significant difference compared with the Met group; $^{d}P < 0.05$ significant difference compared with the Met + D-gal group. D-gal; d-galactose, Met; metformin, BADGE; Bisphenol A diglycidyl ether.

Table 4: Hepatic lipid peroxidation and total antioxidant levels in the experimental groups

Parameters	Experimental groups				
	Control	D- gal	Met	Met + D- gal	BADGE + Met + D- gal
MDA (nmol/mg protein)	12.63±1.33	50.21±2.46 [a]	9.46 ± 0.98 [a,b]	16.56±1.62 [a,b,c]	47.31±3.35 [a,b,c,d]
TAC (ng/mg protein)	18.08±1.73	4.55±1.33[a]	23.61±1.84 [a,b]	14.46±1.65 [a,b,c]	6.66 ± 1.21 [a,b,c,d]

Data are expressed as mean ± standard deviation SD; $n = 6$, [a] $P < 0.05$ significant difference compared with the control group; [b] $P < 0.05$ significant difference compared with the D-galgroup; [c] $P < 0.05$ significant difference compared with the Met group; [d] $P < 0.05$ significant difference compared with the Met + D-galgroup. D-gal; d-galactose, Met; metformin, BADGE;Bisphenol A diglycidyl ether, MDA; malondialdehyde, TAC; Total antioxidant capacity.

Table 5: Hepatic PPAR-γ and irisin protein levels

Parameters	Experimental groups				
	Control	D- gal	Met	Met + D- gal	BADGE + Met + D- gal
PPAR-γ (ng/mg protein)	6.35±0.26	1.72±0.3[a]	7.57±0.27 [a,b]	5.23±0.29 [a,b,c]	3.92±0.14 [a,b,c,d]
Irisin (ng/mg protein)	24.68±1.36	8.01±1.44 [a]	27.67 ± 2.5 [a,b]	19.9 ±1.02 [a,b,c]	9.01 ± 1.43 [a,b,c,d]

Data are expressed as mean ± standard deviation SD; $n = 6$, [a] $P < 0.05$ significant difference compared with the control group; [b] $P < 0.05$ significant difference compared with the D-gal group; [c] $P < 0.05$ significant difference compared with the Met group; [d] $P < 0.05$ significant difference compared with the Met + D-gal group. D-gal; d-galactose, Met; metformin, BADGE; Bisphenol A diglycidyl ether, PPAR-γ; Peroxisome proliferator-activated receptor gamma

3.4. Effect of D-gal and Met administration on hepatic lipid peroxidation and TAC in the experimental groups

Data shown in Table 4 revealed the significant D-gal induced oxidative stress and the beneficial antioxidant effects of Met against D-gal. A remarkable MDA rise with a dramatic TAC decline was reported as a response to D-gal administration in comparison to the control group ($P < 0.05$). Concurrent Met treatment offsets the D-gal action regarding the MDA and TAC. However, the BADGE + Met + D-gal group displayed a notable decline versus the levels observed for the Met + D-gal group while still significantly higher than their corresponding's in the D-gal group ($P < 0.05$).

3.5. Effect of D-gal and Met administration on hepatic protein levels of PPAR-γ and irisin in the experimental groups

Data presented in Table 5show an outstanding decline in hepatic PPAR-γ and irisin protein levels as a response to D-gal administration in comparison to the control group ($P < 0.05$). This confirmed the presence of induced aging-associated PPAR-γ and irisin reduction. Concurrent Met treatment offsets the D-gal-induced action regarding the abovementioned parameter. However, the BADGE + Met + D-gal group displayed observable fading *versus* the levels observed for the Met + D-gal group while still meaningfully higher than their corresponding in the D-gal group ($P < 0.05$).

3.6. Effect of D-gal and Met administration on the hepatic immunohistochemical expression of SIRT1 in the experimental groups (Fig. 3)

Immunohistochemical analysis of hepatic expression of SIRT1 protein showed the immunoreactive basal expression in the control group (Fig. 3A). An increase in the SIRT1 expression was seen in the Met group (Fig. 3C). The D-gal-treated group revealed a marked decline or faint immunohistochemical staining (Fig.3B). D-gal distinctly, down-regulated the SIRT1 expression in the D-gal group when compared to the control group (Fig. 3F). On the other hand, an observed augmented expression of SIRT1 was reported in the Met + D-gal group (Fig. 3D) compared to the D-gal-treated rats (Fig. 3F). Moreover, when Met administration was preceded by BADGE, it caused moderate SIRT1 expression (Fig.3E) compared to Met + D-gal (Fig. 3F).

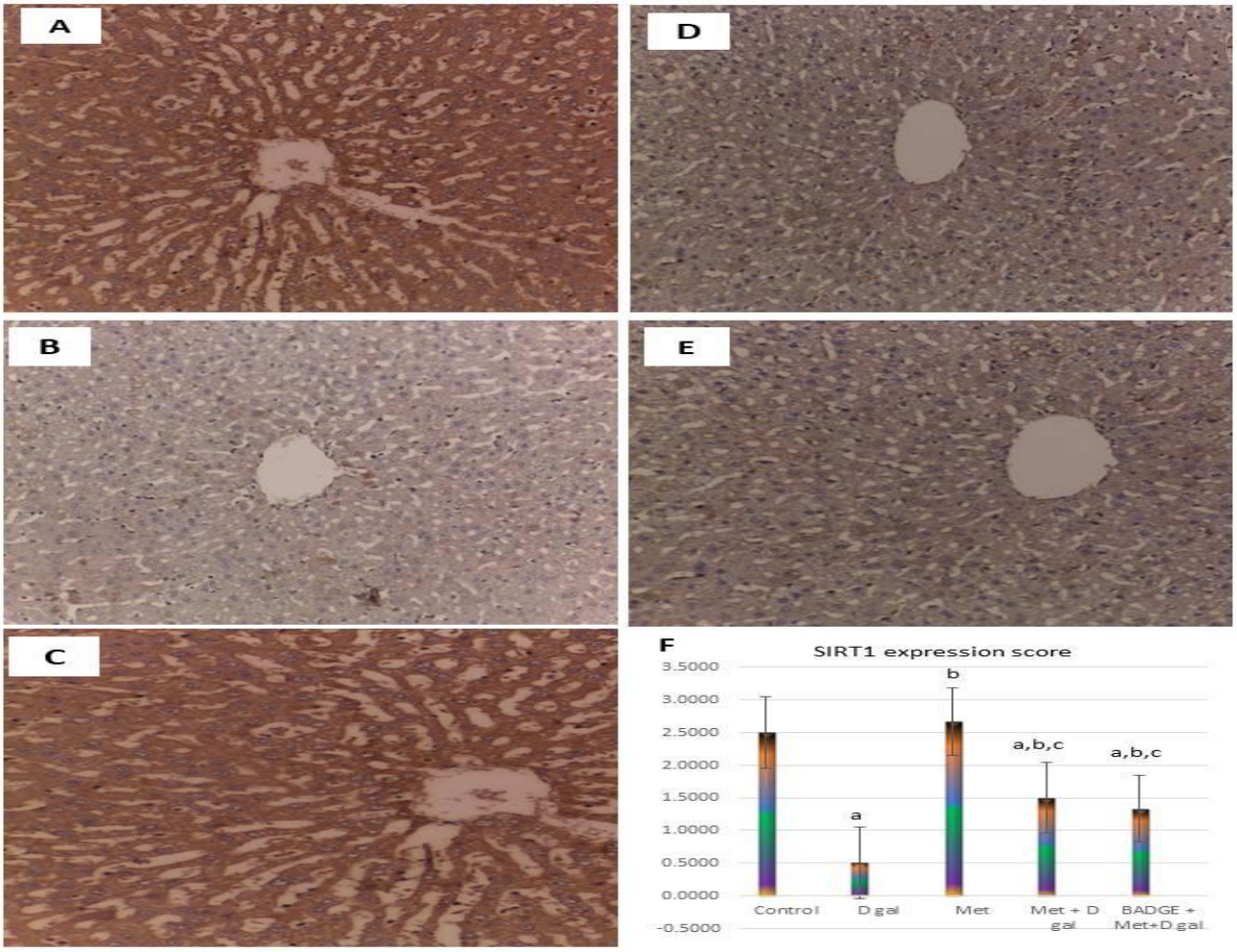

Fig. 3: Immunohistochemical expression of SIRT1 in the experimental groups.

The positive SIRT1 expression is indicated by the brown color of the cytoplasm. (3-A): control group shows the basal expression of SIRT1, (3-B): D-gal group reveals a minimal or faint expression of SIRT1, (3-C): Met group shows strong SIRT1 expression, (3-D): Met + D-gal group shows moderate expression. (3-E): BADGE + Met + D-gal group shows lower expression. Magnification= X200. (3-F): scorning of the SIRT1 expression. Data are expressed as mean ± standard deviation SD; n = 6, [a] $P < 0.05$ significant difference compared with the control group; [b] $P < 0.05$ significant difference compared with the D-gal group; [c] $P < 0.05$ significant difference compared with the Met group; [d] $P < 0.05$ significant difference compared with the Met + D-gal group. D-gal; d-galactose, Met; metformin, BADGE; Bisphenol A diglycidyl ether

3.7. Correlation between irisin and ALT, HOMA-IR, MDA, and TAC parameters (Fig. 4)

There is an observable negative correlation between irisin with ALT ($r = - 0.98$; $p < 0.05$) and HOMA-IR ($r = - 0.84$; $p < 0.05$) besides MDA ($r = - 0.95$; $p < 0.05$). Counteract, a positive correlation with TAC ($r = 0.96$; $p < 0.05$).

4. Discussion

The main findings in the current study were that Met exerted a hepato-protective effect in aged rats. Such an effect was mediated by preservation of redox homeostasis and an abundance of SIRT1/PPAR-γ/irisin molecules pathway. Blockade of PPAR-γ activity significantly reduced irisin formation and partly reduced such effect.

A mounting group of evidence implicates oxidative stress as a crucial aspect of natural hepatic aging pathophysiology [7].Experimentally; chronic D-gal administration can mimic natural aging in rats [3]. High D-gal concentrations produce a torrent of free radicals with a reduction of the TAC. This is due to its conversion into aldose and hydroperoxide with a considerable amount of the most hazardous hydroxyl radical [27]. In addition, there was formation of advanced glycation end products with oxidative potency. Such ROS initiate oxidative damage and promotes cell membrane lipid peroxidation, thus increasing serum levels of MDA, cellular protein denaturation, mitochondrial disruption, and DNA damage. Unfortunately, since the liver is the primary organ concerned with detoxification and metabolism, it becomes the leading attack target of ROS and its consequential complications.

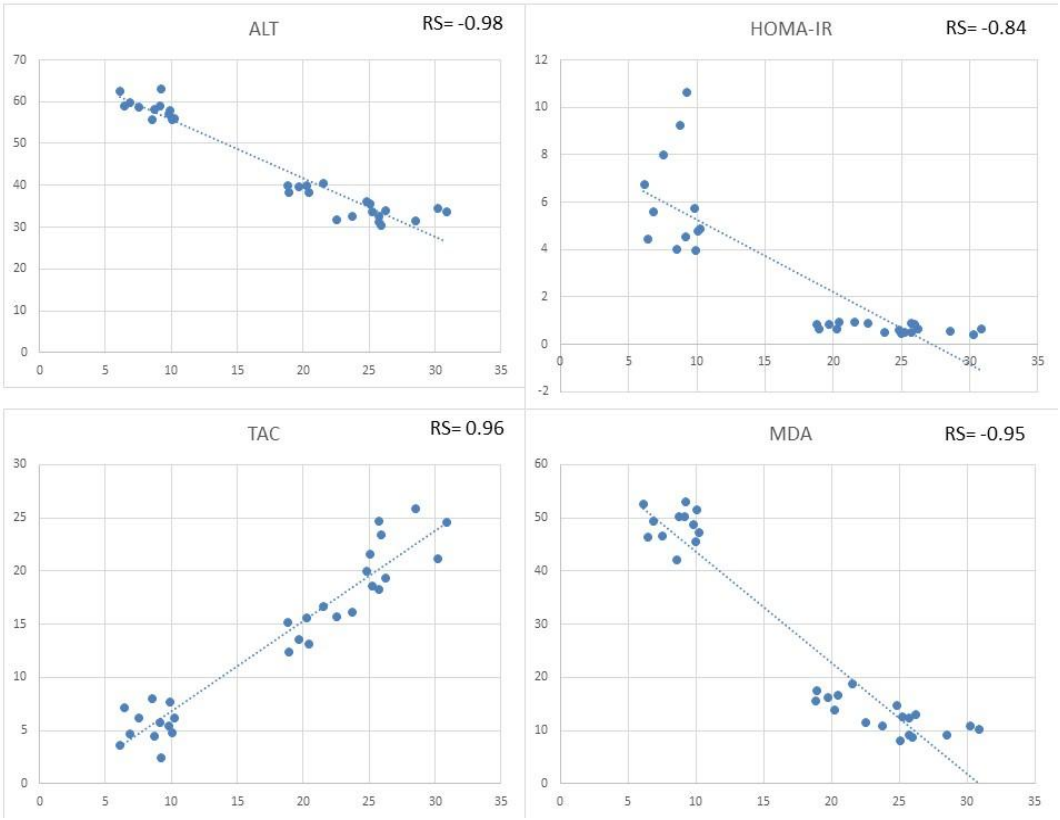

Fig. 4: The correlations of irisin with ALT, HOMA-IR, TAC, and MDA levels.
It was analyzed using Pearson's correlation coefficient (r) 2-tailed test. A p-value of less than 0.05 was considered statistically significant.ALT: Alanine aminotransferase; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance; MDA: Malondialdehyde; TAC: Total antioxidant capacity; Rs: spearman correlation coefficient.

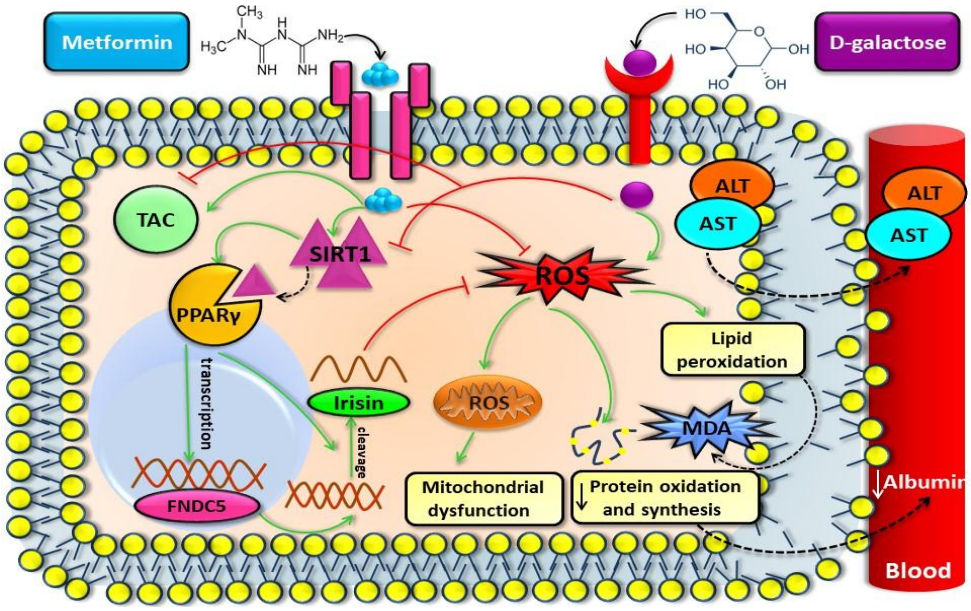

Fig.5: A schematic diagram summarizes the proposed mechanisms of the protective effect of Met against D-gal-induced hepatic aging.
In the liver, Met exerts its effect via 2 pathways: the ROS-quenching and SIRT1/ PPAR-γ/ irisin pathways. It mitigates MDA while enhancing TAC. Furthermore, Met promotes the up-regulation of SIRT1 and facilitates its binding to PPAR-γ. Consequently, this SIRT1/ PPAR-γ complex boosts the proteolytic cleavage of FNDC5 and increases irisin. Irisin counteracts the oxidative pathway and promotes its protective effect. D-galactose causes hepatic cell membrane disruption with leakage of hepatic enzymes and reduced albumin synthesis. ALT; Alanine aminotransferase, AST; Aspartate aminotransferase, FNDC5; fibronectin type III domain 5, MDA; malondialdehyde, PPAR-γ; Peroxisome proliferator-activated receptor gamma, ROS; reactive oxygen species,SIRT1; Sirtuin 1,or Silent information regulator 1, TAC; Total antioxidant capacity. The illustration was created using the software: PowerPoint.

Such a sequel was affirmed in our model by an observable increase in liver MDA, while a decline in the TAC in D-gal administrated group compared to the control. Moreover, hepatocytes membranes disintegration resulted in a leakage of AST and ALT. In this regard also, there was a substantial decline in hepatocytes' protein-synthesizing function resulting in a significant serum albumin reduction. These results were in parallel with others [9, 28-30].

Importantly, the current histopathological findings were in line with the biochemical functional parameters that ensure D-gal-induced hepatic aging. Hepatocellular necrotic and fatty changes with evident inflammatory cell infiltration were remarkable in the D-gal treated group with a significant increase in the hepatic injury score in comparison to the control group. Also, areas of bridging fibrosis were observed. These changes eventually reduce the number and size of fenestrations, cause loss of insulin receptors, and impair its signaling pathways. Subsequently, these events diminished glucose uptake and exacerbated insulin resistance [9]. Our findings go parallel with this ascertain and revealed a significant increase in serum glucose, insulin, and HOMA-IR. These results are consistent with those obtained by Ahangarpour and his collaborators (2018) and Omidi's research team (2020) [31, 32].

Recently, medications with antiaging properties have gained great interest, especially with aging-associated frailty, feeding alterations, and environmental stresses. The present results revealed that combined Met and D-gal

administration prevents such D-gal-induced hepatic aging changes and preserved hepatic functions and structures at both biochemical and histopathological levels when compared to the D-gal-treated group. Thus, Met can introduce itself as a geriatric rehabilitating drug. These results are in accordance with previous studies [10, 33-35].

Since oxidative stress is critical for aging pathogenesis, we next examined the antioxidant potency of Met. A remarkable decline in hepatic MDA, while an increase in TAC levels was documented in the Met + D-gal treated rats versus the D-gal group. This could be explained by Met`s ROS-suppressing activity as a sequel of the diminution of the NADPH oxidase activity [8]. Our findings are consistent with those of Sadeghi et al. who reported Met`s ROS-quenching activity against bile duct ligation–induced hepatic oxidative injury [36]. Also, another study carried out by Yuan et al. [37] revealed the potential role of Met in attenuating endotoxin-induced fulminant hepatic oxidative injury.

Although the mechanisms by which Met targets fundamental pathways in biological aging are far from completely understood, our study attempts to elucidate an innovative mechanism in parallel to the antioxidant pathway by which Met protects against D-gal-induced hepatic aging.

SIRT1, a target agent in aging, modulates transcriptional factors linked to higher expectancy [8] PPAR-γ plays an important role in regulating these vital cellular processes as well as energy homeostasis and oxidant balance [5]. Irisin is a hormone that was recently discovered. It is a fibronectin type III domain 5 (FNDC5) cleavage protein secreted by tissues such as the brain and liver. Furthermore, it plays a role in liver metabolism [38]. Thus, the SIRT1/PPAR-γ/Irisin pathway was supposed as a suggested mechanism underlying the hepatoprotective impact of Met.

In this regard, the current study revealed the existence of a considerable rise in the immunostaining data of SIRT1 expression and PPAR-γ, besides, a significant rise in irisin level in liver tissue following Met administration with D-gal versus D-gal group. The possible explanation is that Met up-regulates SIRT1, either directly or by phosphorylating AMPK, then SIRT1 binds to its binding site in PPAR-γ, causing its activation and up-regulating PPAR-γ [5]. Afterward, PPAR-γ enhances the transcription and proteolytic cleavage of FNDC5 [39] and releases irisin.

These results were consistent with that of Song et al. [40], who found that Met alleviates hepato-steatosis by restoring SIRT1. Also, another study carried out by Mansour and Galal [41] demonstrated the potential role of Met in cisplatin-induced hepatotoxicity *via* up-regulation of PPAR-γ. Also, a related study was conducted by Li et al. [42] who revealed an elevation in irisin level with Met administration.

On the other hand, our findings regarding the D-gal group revealed a significant reduction in hepatic SIRT1 as well as PPAR-γ. This PPAR-γ reduction hindered FNDC5 cleavage and resulted in a significant decrease in irisin level. This irisin decline exacerbated injury to the aged hepatocytes. These data shed light on the hepato-protective impact of irisin during aging. The molecular insights involve regulating several vital cellular processes, comprising mitochondrial functions modulation, autophagy induction, suppressing endoplasmic reticulum stress and inflammasome, inhibiting ferroptosis, and reversing cell death. All

these impacts are dependent on the antioxidant effect of irisin [43].

In the current study, to prove a PPAR-γ-dependent pathway underlying the Met-induced irisin rise, BADGE, a specific PPAR-γ blocker [44], was used. Interestingly, by comparing the assessed parameters in the BADGE + Met + D-gal group to their corresponding in Met + D-gal one, a notable worsening in the hepatic and glucose state was observed. This denoted the blockage action of BADGE and PPAR-γ/ irisin pathway affection that stems a regression in the Met prophylactic influence when co-administered with BADGE.However, when the BADGE-treated group was compared with the D-gal group, we found that the hepatic state was much worsened in the D-gal group. This data shed light on the direct antioxidant potency of Met that possibly, in part, underlying its anti-aging effect besides the SIRT1/ PPAR-γ/ Irisin pathway.

An illustration of the underlying mechanisms involved in the hepato-protective impact effect of Met was showed in Fig. 5.

Conclusion

The current research revealed that Met could indeed enhance prophylaxis against senescence-induced hepatic dysfunctions and insulin resistance. It blunted hepatocellular changes at both structural and functional aspects. This gero-protective impact was achieved by conserving the redox and glucose homeostasis in addition to boosting the SIRT1/PPAR-γ/irisin signalling cascade.A mechanism that was, in part, dependent on PPAR-γ as evidenced after its blockade by BADGE. Thus, persons on Met medication are more likely to have a lower incidence of age-related hepatic insults.Due to slowing the aging process, blunting its associated bad consequences, and old decades of safety, Met appeared to be a promising anti-aging pill.

Declarations:

Data availability: Upon request, the corresponding author will provide the data that support the findings of this study.

Compliance with ethical standards: The study was executed by following the rules and guidelines of the Institutional Animal Ethics Committee, Benha University, Egypt(Approval protocol numberBUFM11072020).

Author contributions: All authors contributed equally to the study design, collection of samples, data analysis, and manuscript writing.

Competing interest: The authors reported no potential conflicts of interest.

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