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Research Article

Alagebrium Mitigates Metabolic Insults in High Carbohydrate and High Fat Diet Fed Wistar Rats

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Abstract

Background: Metabolic syndrome (MS) is characterized by sustained hyperglycemia that triggers advanced glycation end products (AGEs) generation. Alagebrium (ALA) is an advanced glycation end products (AGEs) cross-links breaker.

Methods: 32 Wistar rats were divided into normal control (NC) group (8 rats) and MS groups (24 rats) received a high carbohydrate high fat diet (HCFD) for 10 weeks. Rats with established MS were equally divided into 3 subgroups remained on HCFD for further 6 weeks: MS control (MSC), ALA treated received 10 mg/kg/day ALA orally and metformin treated (MF) (a reference drug) received 50 mg/kg/day MF orally. The studied parameters were systolic blood pressure (SBP), body and liver weights (BW, LW), LW/BW% ratio, fasting blood glucose (FBG), serum insulin, lipid profile, liver enzymes, serum AGEs, hepatic Interleukin-17 (IL-17), adipokines, pAkt/Akt ratio, and liver histopathology.

Results: HCFD elevated SBP, BW, LW and LW/BW% ratio, FBG, serum insulin, and AGEs. It also deteriorated lipid profile and liver enzymes, induced inflammation, insulin resistance and histopathological derangements. ALA ameliorated the elevated SBP, FBG, lipid profile, liver enzymes, mitigated insulin resistance, hepatic IL-17, serum AGEs, modulated adipokines levels and improved liver histopathology. However, MF had better effects than ALA in all studied parameters except AGEs.

Conclusion: ALA is protective against dietary-induced MS via ameliorating the inflammatory process and serum AGEs that implicated in MS pathogenesis, which makes it a promising new tool in MS treatment.

Introduction

Metabolic syndrome (MS), a major challenge for public health, obtained a significant importance because of the exponential increase in the worldwide obesity that caused mainly by sedentary lifestyle habits and excessive intake of calories from a diet rich in fats and sugars.[1,2] MS refers to a collection of many metabolic abnormalities including; hypertension, central obesity, insulin resistance, and dyslipidemia.[3]

Chronic sustained hyperglycemia triggers the formation of advanced glycation end products (AGEs), which are formed through a connection between sugar and protein by covalent bonds via a non-enzymatic process.[4] These AGEs are implicated in the pathogenesis of diabetes mellitus, MS and insulin resistance.[5] AGEs mediate their pathological effects through activation of signaling cascades after binding to the receptor for advanced glycation end products (RAGE).[6]

The insulin resistance (IR), an important pathological feature of MS,[7] occurs because of the defective insulin action both in the liver and in muscle.[8] This IR, associated with concomitant hyperinsulinemia, may potentiate other MS components such as hypertriglyceridemia and fatty liver.[9] One of the major downstream insulin receptor substrate 1 (IRS1) targets is Akt (protein kinase B), an important mediator of cell survival, proliferation, angiogenesis, which has been implicated in numerous metabolic actions of insulin after being phosphorylated.[9] Akt also phosphorylates a pivotal regulatory molecules in glucose metabolism,[10] and phosphorylation of some of these regulatory substrates is defective in cases of IR.[10] Other pathophysiological mechanisms associated with MS and obesity are the adipocytokines, signaling factors overproduced from the hypertrophied adipocytes,[11] such as pro-inflammatory interleukin-6 (IL-6),[12] interleukin-17 (IL-17)[13] and tumor necrosis factor -alpha (TNF-α).[14] Furthermore, Adipocyte dysfunctions impair the secretion of adiponectin which plays important roles not only in appetite and satiety regulation but also in insulin sensitivity and secretion, inflammation, expenditure of energy and endothelial function.[15]

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Alagebrium (ALA) was the primary drug originally introduced to break the covalent bonds formed in cross-linked proteins, in order to maintain the normal function of these proteins after being detached, and even if re-bonding occur, ALA can also break up these bonds again, so reducing the tissue levels of AGEs and ameliorating their hazardous effects.[16,17]

Metformin (MF) the most commonly prescribed drug for diabetes mellitus type 2 (DM-2), works by decreasing absorption of glucose from the intestine, enhancing peripheral glucose uptake, lowering fasting plasma insulin and enhancing insulin sensitivity,[18] leading to the reduction in blood glucose without causing hypoglycemia**,** MF can also inhibit gluconeogenesis through the activation of AMP-activated protein kinase (AMPK), which is an important energy metabolism regulator.[19] Besides, the glucose-lowering action of MF, it improves other features of MS, not only by improving the insulin sensitivity in liver and muscle[18] but also by its beneficial hypolipidemic, antioxidant, cardio-protective and anti-inflammatory actions.[20]

Management of metabolic syndrome requires novel pharmacological approaches to ameliorate the pathogenic mechanisms implicated in the disease development and progression. So, this study aimed to investigate the possible protective effects of ALA as a monotherapy agent on the sequelae of MS in Wistar rats and also to outline its possible underlying mechanisms.

Materials and Methods

Animals

Adult male Wistar rats (180–200 g) were purchased from Zagazig University Center of Experimental Animals, Faculty of Veterinary Medicine. The rats were left for acclimatization one week before the experiment under specific pathogen-free conditions; with 12-h light/dark cycles at a temperature of 23±2 °C. The experiments were conducted at the Animal House of Zagazig Faculty of Medicine.

Drugs

Alagebrium chloride (cat. no. 341028-37-3) was purchased from CHEMOS GmbH & Co. KG (Regenstauf, Germany). Metformin hydrochloride was purchased from Minipharma for Pharmaceutical & Chemical Industries, (10th of Ramadan – Egypt, under license of Merck Sante, France)**.** All other chemicals used in this study were of analytical grade obtained from Sigma/Aldrich unless otherwise noted.

Experimental study design and drug treatment

Thirty-two adult male Wistar rats were randomly assigned into two initial groups: (I) Normal control group (NC) (8 rats) for 16 weeks the animals received an ordinary standard chow diet (A04, SAFE, Augy, France) and distilled water (II) metabolic syndrome groups.

Metabolic syndrome groups

24 rats were maintained on high carbohydrate and high fat diet (HCFD) which is composed of condensed milk (39.5%), beef tallow (20%), and fructose (17.5%) together with 25% fructose in drinking water for 16 weeks.[21] HCHF diet consists of 175 g fructose, 395 g condensed milk, 200 g beef tallow, 155 g standard diet and 50 g of water per each kilogram of diet in addition to 25% fructose in the drinking water. Calories of the total energy intake were 4.26 kcal/kg; with 0.92 kcal/mL fructose in drinking water. These total calories were calculated from the following values: fructose (3.68 kcal/g), condensed milk (3.29 kcal/g), beef tallow (9.01 kcal/g) and standard diet (3.29 kcal/g).[21] Food and water were given ad libitum to rats. To confirm the induction of MS, the collection of hyperglycemia, hypertension, hyperlipidemia (elevation of triglycerides and total cholesterol) and obesity were established in animals fed HCFD. As after 10 weeks of HCFD feeding, the blood glucose level was estimated after 12 hours fasting by using blood glucose test strip (BIONIME Corporation, Taiwan), the systolic blood pressure (SBP) was measured by using 8-Channel Non-Invasive Blood Pressure Monitor (Columbus Instruments, USA) after rat acclimatization and without anesthesia, the tail of the rat was completely out the hole in the back of the restrainer where a cuff with a pneumatic pulse sensor was attached (the cuff pressure was set to 250 mmHg) and 7-5 readings were recorded for each rat, with 2-3 min intervals and the mean value of the last five measurements was used, rats body weights were measured by digital weight scale as an index of obesity and triglycerides and total cholesterol were estimated by the quantitative colorimetric determination method (Elabscience, USA) after collection of blood samples from the retro-orbital plexus under light anesthesia then the blood was processed rapidly into serum (centrifuged at 604 g for 10 minutes) to confirm the elevation of these lipid parameters.

Rats with established MS were further divided into 3 subgroups (8 rats each) to be remained on HCFD for further six weeks: metabolic syndrome control group (MSC): received distilled water (0.3 ml/100 g/day), alagebrium treated group (ALA): received 10 mg/kg alagebrium chloride,[22] metformin treated group (MF): received metformin hydrochloride (50 mg/kg/day).[23] All drugs were dissolved in distilled water and given orally by gavage once-daily dose for 6 weeks.

Animal sacrifaction and blood pressure monitoring

The dose of ALA was selected according to the dose used in previous experimental studies.[22,25-27] MF dose was selected according to the pharmacokinetic formula for humans and rats.[24] At the end of the experimental study the body weights of rats were estimated, and also SBP estimation was done by the Power Lab (4/35) data acquisition system (Australia) after anesthetizing rats with an intraperitoneal injection of pentobarbital (60 mg/kg, Sigma, St. Louis, USA).[28,29] Carotid cannulation was done in which one end

of the cannula was into the carotid artery however the other was connected to a three-way stopcock. The pressure transducer was connected to this three-way stopcock together with a heparinized saline-filled syringe that helped the application and maintenance of positive pressure at the baseline level. The three-way stopcock acts as a bridge to connect the pressure transducer and the carotid cannula. Recording of the blood pressure was done by converting the electrical signal of the pressure transducer unit.[30] After recording the blood pressure, blood samples were collected in clean dry test tubes from the inserted arterial polyethylene carotid artery cannula after that rats sacrificed by decapitation.

Sampling of blood and tissue

Immediately after sacrification, the blood samples were separated by centrifugation at 604 g for 10 minutes to get clear sera. Livers were excised and rapidly rinsed with saline, then weighted by digital weight scale to estimate the liver weight body weight ratio (LW/BW % ratio), then each organ was divided equally into two parts; one part was homogenized for bioassay of IL-17, pAkt/Akt, leptin and adiponectin; and the other one was preserved for histopathological examination in 10% formalin. In all the estimated biochemical parameters, either in serum or tissue, the manufacturer's protocol was followed.

Liver tissue homogenates

Liver tissue was rinsed, then homogenized by Mixer Homogenizer (Thomas Scientific, USA) in 1 ml buffered phosphate solution(BPS) for each 100 mg/tissue and stored overnight at -20 °C. Two freeze-thaw cycles were performed to break the cell membranes, after that the homogenates were centrifuged at 5000 g, 2-8 °C for 5 minutes. The samples were stored at -80 °C. Before the assay centrifugation of the sample was done again after thawing before.

Glucose metabolism determinations

Fasting blood glucose (FBG) concentrations were measured after 12 hrs fasting by using glucose-oxidase enzymatic commercial kit (catalog no. GOD-POD, Spinreact SAU, Sant Esteve de Bas, Spain) the intra-assay CV is 0.48 % and the inter-assay CV is 2.57 %. Serum insulin levels were measured by using an ultrasensitive insulin enzyme immunoassay commercial kit for rats (catalog no. 10-1113-01, Mercodia AB, Uppsala, Sweden) the intra-assay CV is 3.4 % and the inter-assay CV is 3.6 %. Then, both FBG and insulin concentrations were used to calculate the index of insulin resistance (homeostasis model assessment [HOMA]-IR) according to this formula:

HOMA IR = serum insulin (μIU/ml)×(blood glucose (mg/dl)/405.[24,25]

Serum total cholesterol and triglycerides determination

Quantitative colorimetric determination method was used to estimate serum levels of total cholesterol (Catalog No: E-BC-K109-S, the intra-assay CV is 1.1% and the inter-assay CV is 2.8%) and triglycerides (Catalog No: E-BC-K261-S, the intra-assay CV is ≤5.0% and the inter-assay CV ≤8%) (Elabscience, 14780 Memorial Drive, Suite 216, Houston, Texas, USA).

Estimation of serum aspartate aminotransferase (AST) and alkaline phosphatase (ALP)

The quantitative determination method of serum AST (catalog No. A559-150, TECO Diagnostics, USA) was used by a coupled reaction of malate dehydrogenase (MDH) and NADH. AST catalyzes the transfer of an amino group between L-aspartate and 2-oxoglutarate and the formed 2-oxoglutarate is then reacted with NADH in the presence of MDH to form NAD. The activity of AST was determined by measuring NADH oxidation rate at absorbance rate 340 nm. The intra-assay CV is 2.1% and the inter-assay CV is 1.9%. ALP (catalog No. A504-150, TECO Diagnostics, USA) quantitative determination method in serum was used, as ALP was determined by measuring the rate of hydrolysis of p-Nitrophenyl phosphate (NPP) ester that was used as a substrate. The enzymatic sequence employed in the assay was

p-Npp + H_2O -------->p-Nitrophenol + H_3PO_4.

P-Npp is colorless but p-Nitrophenol has strong absorbance at 405 nm. The rate of increased absorbance at 405 nm is proportional to the enzyme activity. The intra-assay CV is 2.2% and the inter-assay CV is 2.14 %.

Assay of the inflammatory marker interleukin- 17 (IL-17) in liver tissue extract

Hepatic IL-17 (Catalog No. CSB-E07451r, CUSABIO, USA) activities were evaluated by the quantitative enzyme immunoassay technique. IL-17 specific antibody has been pre-coated onto a micro-plate. The wells were pipette by both standards and samples where the antibody bound to any IL-17 present. Removal of any unbound substances was done and a biotin-conjugated antibody specific for IL-17 was added after that avidin conjugated Horseradish Peroxidase (HRP) was added to the wells. After that, a color developed in proportion to the amount of IL-17 bound in the first step after adding a substrate solution and the color intensity was measured. The intra-assay precision CV% is <8 % and the inter-assay precision is CV% <10 %.

Assay of leptin and adiponectin levels in liver homogenates

Leptin (Catalog No. CSB-E07433r, CUSABIO, USA) and adiponectin (Catalog No. CSB-E07271r, CUSABIO, USA) in liver tissue were measured by the quantitative sandwich enzyme immunoassay technique but the antibody specific for each one of them was different. The antibody has been pre-coated onto a microplate and any adipokine present was bound to it with the removal of any unbound substances, then a biotin-conjugated antibody specific for the adipokine was added. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added. Following the removal of any unbound avidin-enzyme reagent, a substrate solution

was added and color develops proportionally to the bound adipokine amount. The color intensity was measured as the microplate reader was set at 450 nm. For both leptin and adiponectin, the Intra-assay Precision CV% is <8 % and the Inter-assay Precision is CV% <10 %.

Assay of serum AGEs levels

Measurement of serum AGEs (catalog No. ABIN368041, antibodies, Germany) was performed with a competitive sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). An antibody specific to AGEs has been pre-coated onto a microplate. A reaction between biotin-labeled and unlabeled AGE (either for standards or samples) with the pre-coated antibody specific to AGE was started. The unbound conjugate was washed off after incubation. After that, to each microplate, avidin conjugated to HRP was added then incubated. Removal of unbound avidin-enzyme reagent was done, then a substrate solution was added and the color developed was proportionate to the amount of bound AGEs and the intensity of the color was measured at 450 nm by ELISA microplate reader. The intra-assay precision of serum AGEs Kits is ≤ 8 %, however, the inter-assay precision is ≤ 10%.

Assay of p-Akt/Akt ratio in liver tissue extract

Phosphorylated Akt [pS473] (catalog No. EIA-3997, DRG International, Inc. USA) was estimated by a sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) as a monoclonal antibodies specific for pAkt have been coated onto the wells where the samples, including a standard containing Akt [pS473], unknowns and control specimens, were pipetted. Then Akt antigen bound to the immobilized antibody after their incubation. After that washing was done then a specific antibody for phosphorylated Akt was added to the wells, which served as a detector by binding to the immobilized Akt protein. Then excessive detection antibody was removed and an anti-rabbit IgG-HRP was added to complete the four-member sandwich. After incubation for a third time, a substrate solution was added, to act upon by the bound enzyme, producing color.

The intensity of this product color is proportional to Akt [pS473] concentration found in the original specimen. The absorbance of each well at 450 nm was determined. The total Akt by a sandwich ELISA (catalog No. EIA-3996, DRG International, Inc. USA) was done in the same way of phosphorylated Akt [pS473] assay except the biotin-conjugated monoclonal antibody was specific for total Akt (the intra-assay precision is 6.3 %, however, the inter-assay precision is 8.7%). Then phosphorylated Akt /Akt ratio (pAkt/Akt) was determined.

Liver histopathology

Specimens of the liver were fixed in formalin 10%; then embedded in paraffin wax. Sections were cut at a thickness of 4 µm and stained using: Hematoxylin and Eosin (H&E) and Mallory Trichrome (MT) stains. Under the light microscopy, the slides were examined to evaluate the severity of NAFLD and the degree of fibrosis, the histopathology scoring was performed using the method described by Kleiner *et al.*[33] (Table 1).

Statistical analysis

One way analysis of variances (one-way ANOVA) was used to evaluate the statistical difference between groups followed by post hoc least significant difference (LSD) for multiple comparisons. The differences were considered significant when $p < 0.05$. Data were expressed as mean ± SD. Kruskal-Wallis followed by Mann-Whitney's test

Table 1. Non-alcoholic fatty liver disease (NAFLD) Activity Score (NAS).

Non-alcoholic fatty liver disease (NAFLD) Activity Score (NAS) Components		
Item	**Score**	**Extent**
Steatosis	0	<5%
	1	5-33%
	2	>33-66%
	3	>66%
Lobular inflammation	0	No foci
	1	< 2 foci
	2	2-4 foci
	3	> 4 foci
Ballooning of the hepatocyte	0	None
	1	Few cells with ballooning
	2	Prominent ballooning
The Stage of Fibrosis (to be separately evaluated from NAS)		
Fibrosis	0	None
	1	Perisinusoidal or periportal
	1A	Mild, zone 3, perisinusoidal
	1B	Moderate, zone 3, perisinusoidal
	1C	Portal/periportal
	2	Perisinusoidal and portal/periportal
	3	Bridging fibrosis
	4	Cirrhosis

Table 2. Effect of a single daily oral dose of 10 mg/kg alagebrium and 50 mg/kg metformin for 6 weeks on BW, LW, LW/BW ratio, SBP, FBG, serum insulin, and HOMA-IR in male Wistar rats with metabolic syndrome.

Groups (n=8)	BW (g)	LW (g)	LW/BW ratio (%)	SBP (mm/Hg)	FBG (mg/dl)	Serum insulin level (µIU/ml)	HOMA-IR
NC group	337±12.34^{A}	8.8±1.06^{A}	2.6±0.31^{A}	101±7.82^{A}	93.5±6.12^{A}	2.5±0.21^{A}	0.57±0.03^{A}
MSC group	407±14.47^{B}	13.8±0.73^{B}	3.3±0.22^{B}	145 ±5.44^{B}	167.6±5.83^{B}	13.2±1.15^{B}	5.46±0.62^{B}
ALA group	396±11.43^{B}	12.9±1.34^{B}	3.2±0.38^{B}	121 ±2.46^{C}	134±2.82^{C}	8.3±0.50^{C}	2.74±0.18^{C}
MF group	364±13.58^{C}	9.8±1.36^{A}	2.7±0.37^{A}	117 ±3.99^{C}	95.4±10.67^{A}	3.6±0.32^{A}	0.77±0.13^{A}

NC: normal control group; **MSC**: metabolic syndrome control group; **ALA**: alagebrium treated group; **MF**: metformin treated group; **BW**: body weight; **LW**: Liver weight; **LW/BW**: liver weight body weight ratio, **SBP**: systolic blood pressure; **FBG**: fasting blood glucose, **HOMA-IR:** homeostasis model assessment of insulin resistance; **n**: number of rats in each group. Statistical analysis was done by one way ANOVA. Values represent (Mean ± SD). Values within the same column with different superscript capital letters are significantly ($p<0.05$) different compared to each studied group.

were used to analyze histopathological results. Version 14 of SPSS software was used to analyze the results (SPSS Inc, Chicago, USA).

Results

Effect of ALA and MF on body weight (BW)

The BW was significantly higher in the MSC group than the NC group (407±14.47 g vs. 337±12.34 g, $p<0.05$). In ALA group, the BW showed no significant difference compared to the MSC group, on the other hand, it showed a significantly higher value than the NC group (369±11.43 g vs. 337±12.34 g, $p<0.05$). The BW in MF group was 364±13.58 g which was significantly ($p<0.05$) lower compared to both MSC and ALA groups, however, it was significantly ($p<0.05$) higher compared to the NC group (Table 2).

Effect of ALA and MF on liver weight (LW) and LW/BW% ratio

The LW and LW/BW% ratio in the MSC group were significantly higher than the NC group (13.8±0.73 gm and 3.3±0.22 respectively in MSC group vs. 8.8±1.06 gm and 2.6±0.31 respectively in NC, $p<0.05$). The LW and LW/BW% ratio in ALA group were 12.9±1.34 gm and 3.2±0.38 respectively, which were not significantly different compared to the MSC group, on the other hand, they were significantly ($p<0.05$) higher than the NC group. In MF group, the LW was 9.8±1.36 gm and LW/BW% ratio was 2.7±0.37 which were significantly ($p<0.05$) lower when compared not only to the MSC group but also to ALA group, however, both values were not significantly different compared to NC group (Table 2).

Effect of ALA and MF on systolic blood pressure (SBP)

The SBP in the MSC group was significantly higher compared to the NC group (145±5.44 mm/Hg vs. 101±7.82 mm/Hg, $P<0.05$). In ALA group the SBP was significantly lower compared to the MSC group (121±2.46 mm/Hg vs. 145±5.44 mm/Hg, $P<0.05$), on the other hand, it was significantly higher compared to the NC group (121±2.46 mm/Hg vs. 101±7.82 mm/Hg, $P<0.05$). In MF group the SBP was 117 ± 3.99 mm/Hg that was significantly ($p<0.05$) lower compared to the MSC group, however, it was significantly ($p<0.05$) higher compared to the NC group. On the other hand, the SBP value in MF group was not significantly different compared to ALA group (Table 2).

Effect of ALA and MF on FBG and fasting serum insulin

In the MSC group, the FBG and serum insulin showed significantly ($p<0.05$) higher levels compared to the NC group (167.6±5.83 mg/dl and 13.2±1.15 µIU/ml respectively in MSC group vs. 93.5±6.12 mg/dl and 2.5±0.21 µIU/ml respectively in NC group, $p<0.05$). In ALA group the FBG and the serum insulin were 134±2.82 mg/dl and 8.3±0.50 µIU/ml respectively which were significantly ($p<0.05$) lower compared to the MSC group, on contrast, they were significantly ($p<0.05$) higher compared to the NC group. In MF group, the FBG was 95.4±10.67 mg/dl and serum insulin level was 3.6±0.32 µIU/ml which were significantly ($p<0.05$) lower compared to both the MSC and ALA groups with no significant difference in comparison to the group of NC (Table 2).

Effect of ALA and MF on HOMA-IR

HOMA-IR in the MSC group was significantly higher than the NC group (5.46 ± 0.62 vs. 0.57±0.03, $p<0.05$). In ALA group the HOMA-IR was 2.74±0.18 that was significantly ($p<0.05$) higher than the MSC group, on the other hand, it was significantly ($p<0.05$) lower than the NC group. In MF group, the HOMA-IR was 0.77 ±0.13 which was significantly ($p<0.05$) lower compared not only to the MSC group but also to the ALA group, however, this value was not significantly different compared to the NC group (Table 2).

Effect of ALA and MF on serum triglycerides (TGs) and total cholesterol

In the MSC group serum TGs and total cholesterol showed significantly higher levels compared to the NC group (82.6±3.32 and 84.2 ±3.15 mg/dl respectively vs. 36.5±1.68 and 52.6 ±2.32 mg/dl respectively, $p<0.05$). In ALA group, the TGs and total cholesterol levels were 65.4±4.27 and 73±5.19 mg/dl respectively which were significantly ($p<0.05$) lower compared to the MSC group, however, these values were significantly ($p<0.05$) higher than the NC group. In MF group the TGs levels were 40.2±2.76

and the total cholesterol levels were 57.6±4.27mg/dl, these values were significantly (p<0.05) lower in comparison to both MSC and ALA groups, on the other hand, they were significantly (p<0.05) higher than the NC group (Table 3).

Effect of ALA and MF on serum liver enzymes (AST and ALP)

Serum AST and ALP levels in MSC group were significantly higher compared to NC group (63.5±4.65U/L and 111.8±6.08 U/L respectively vs. 24.5±1.41 U/L and 31.4±1.76 U/L respectively, p<0.05). In ALA group serum AST and ALP were 43.2±4.52U/L and 71.5±7.19 U/L respectively which were significantly (p<0.05) lower than MSC group, however, they were significantly (p<0.05) higher compared to NC group. In MF group the serum AST and ALP were 29.2±2.24 U/L and 37.6±4.01 U/L respectively, these values were significantly (p<0.05) lower compared to both MSC and ALA groups, on the other hand, they were significantly (p<0.05) higher compared to NC group (Table 3).

Effect of ALA and MF on hepatic IL-17

In the MSC group, the hepatic IL-17 level was significantly higher compared to the NC group (21.6±1.84 pg/gm tissue vs. 2.7±0.46 pg/gm tissue, p<0.05). The hepatic IL-17 in ALA group was 11.9±1.08 pg/gm tissue, which was significantly (p<0.05) lower than MSC group, on the other hand, it was significantly (p<0.05) higher than NC group. In MF group the hepatic IL-17 was 5.6±1.22 pg/gm tissue, which was significantly (p<0.05) lower compared not only to the MSC group but also to ALA group, however, it was significantly higher than the NC group (p<0.05) (Figure 1a).

Effect of ALA and MF on liver pAkt/Akt ratio

The liver pAkt/Akt ratio in the MSC group was significantly lower compared to the NC group (0.56±0.04 vs. 0.93±0.05, p<0.05). In ALA group the hepatic pAkt/Akt ratio was 0.62±0.02 which was significantly (p<0.05) higher than the MSC group, on the other hand, it was significantly (p<0.05) lower than NC group. In MF group the hepatic pAkt/Akt ratio was 0.92±0.07 which was significantly (p<0.05) higher in comparison to both MSC and ALA groups, however, it was not significantly different compared to NC group (Figure 1b).

Table 3. Effect of a single daily oral dose of 10 mg/kg alagebrium and 50 mg/kg metformin for 6 weeks on serum liver enzymes and lipid profile in male Wistar rats with metabolic syndrome.

Groups (n=8)	Serum lipid profile		Serum liver enzymes	
	TGs (mg/dl)	Total cholesterol (mg/dl)	AST (U/L)	ALP (U/L)
NC group	36.5±1.68^{A}	52.6±2.32^{A}	24.5±1.41^{A}	31.3±1.76^{A}
MSC group	82.6±3.32^{B}	84.2±3.15 ^{B}	63.5±4.65^{B}	111.8±6.08^{B}
ALA group	65.4±4.27^{C}	73±5.19^{C}	43.2±4.52^{C}	71.5±7.19^{C}
MF group	40.2±2.76^{D}	57.6±4.27^{D}	29.2±2.24^{D}	37.6±4.01^{D}

NC: normal control group; **MS**C: metabolic syndrome control group; **ALA**: alagebrium treated group; **MF**: metformin treated group; **TGs**: Triglycerides; **AST**: Aspartate-amino transferase, **ALP:** alkaline phosphatase; **n**: number of rats in each group). Statistical analysis was done by one way ANOVA. Values represent (Mean ± SD). Values within the same column with different superscript capital letters are significantly (p<0.05) different compared to each studied group.

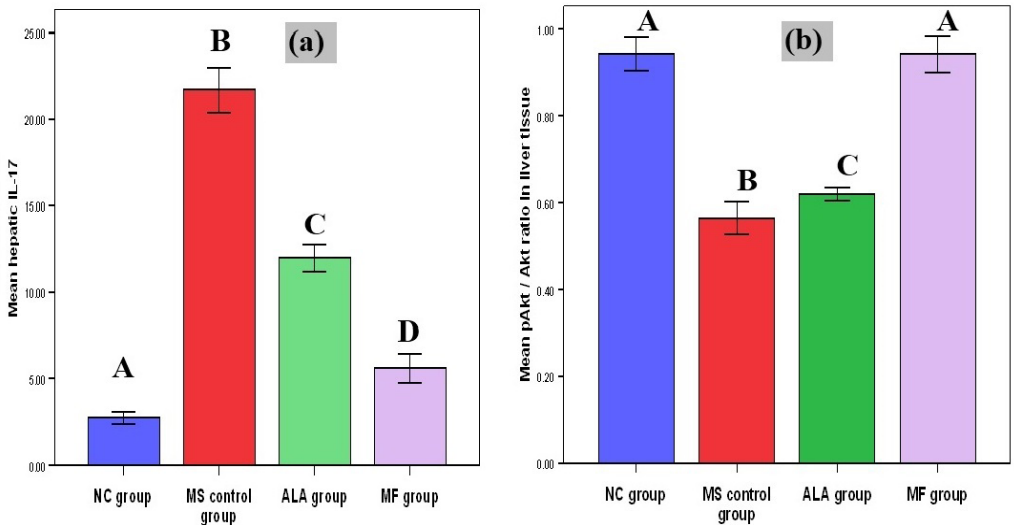

Figure 1. Effect of a single daily oral dose of 10 mg/kg alagebrium and 50 mg/kg metformin for 6 weeks on hepatic (a) IL-17 and (b) pAkt/Akt ratio in male albino rats with metabolic syndrome.
NC: normal control group; **MS**: metabolic syndrome control group; **ALA**: alagebrium treated group; **MF**: metformin-treated group; **IL-17**: interleukin-17; **pAkt/Akt ratio**: phosphorylated Akt/Akt ratio. Values represent (Mean ± SD), different capital letters are significantly (p<0.05) different compared to each studied group.

Table 4. Effect of a single daily oral dose of 10 mg/kg alagebrium and 50 mg/kg metformin for 6 weeks on hepatic levels of leptin and adiponectin, and serum levels of AGEs in male Wistar rats with metabolic syndrome.

Group (n=8)	Adipokine levels		Serum AGEs (U/ml)
	Hepatic leptin (ng/g tissue)	Hepatic adiponectin (ng/g tissue)	
NC group	2.6±0.38^{A}	4.9±0.17^{A}	9.2 ± 1.04^{A}
MSC group	12.5±0.95^{B}	0.7±0.09^{B}	16.4±1.92^{B}
ALA group	6.6±0.78^{C}	1.8±0.16^{C}	11.7±0.76^{C}
MF group	4.7±0.73^{D}	3.7±0.32^{D}	13.1±1.21^{D}

NC: normal control group; **MSC**: metabolic syndrome control group; **ALA**: alagebrium treated group; **MF**: metformin treated group; **AGEs:** advanced glycation end-products; **n**: number of rats in each group. Statistical analysis was done by one way ANOVA. Values represent (Mean ± SD). Values in the same column with different superscript capital letters are significantly ($p<0.05$) different compared to each studied group.

Effect of ALA and MF on hepatic leptin

In the MSC group, the hepatic leptin level was significantly higher than the NC group (12.5±0.95 ng/gm tissue vs. 2.6±0.38 ng/gm tissue, $p<0.05$). In ALA group the hepatic leptin level was 6.6±0.78 ng/gm tissue which was significantly ($p<0.05$) lower compared to MSC rats, however, it was significantly ($p<0.05$) higher than NC group. In MF group the hepatic leptin level was 4.7±0.73 ng/gm tissue which was significantly ($p<0.05$) lower compared to both MSC and ALA groups, on the other hand, it was significantly ($p<0.05$) higher than NC group (Table 4).

Effect of ALA and MF on hepatic adiponectin level

Hepatic adiponectin in the MSC group showed significantly lower levels compared to NC group (0.7±0.09 ng/gm tissue vs. 4.9±0.17 ng/gm tissue, $p<0.05$). In ALA group, the hepatic adiponectin levels were 1.8±0.16 ng/gm tissue which were significantly ($p<0.05$) higher than the MSC group and significantly ($p<0.05$) lower than NC group. In MF group the hepatic adiponectin level was 3.7±0.32 ng/gm tissue which showed significantly ($p<0.05$) higher values compared not only to the MSC group but also to the ALA group, however, it was significantly ($p<0.05$) lower than NC group (Table 4).

Effect of ALA and MF on serum AGEs levels

MSC group showed significantly higher levels of serum AGEs compared to the NC group (16.4±1.92 U/ml vs. 9.2±1.104 U/ml, $p<0.05$). In ALA group, the serum levels of AGEs were 11.7±0.76 U/ml which were significantly ($p<0.05$) lower than the MSC group, however they were significantly ($p<0.05$) higher than NC group. In MF group serum AGEs levels were 13.1±1.21 U/ml that were significantly ($p<0.05$) lower than the MSC group, on the other hand they were significantly ($p<0.05$) higher than the levels estimated in both NC and ALA groups (Table 4).

Histopathology

In the NC group, the NAFLD activity score (NAS) and the stage of fibrosis were 0.13±0.35 and 0±0.0 respectively. In the MSC group, the NAS and the stage of fibrosis were 7.3±0.91 and 1.6±0.51 respectively which were significantly ($p<0.05$) higher compared to the NC group. MSC rats showed marked steatotic changes and ballooning with marked interlobular inflammation with excessive deposition of collagen bands around the central vein and in between the liver cells with aggregated inflammatory cells. In ALA group the NAS and the stage of fibrosis were 4±1.51 and 1.13±0.35 respectively which were significantly ($p<0.05$) higher compared to NC group, on contrast they were significantly ($p<0.05$) lower compared to MSC group. The liver sections of ALA group showed an improvement in the fatty liver changes regarding steatosis and ballooning of the hepatocytes with no interlobular inflammation. Moreover, the liver sections revealed moderate collagen bands around the central vein and in between the liver cells. As regards MF group the NAS and stage of fibrosis were 0.5±0.75 and 0.13±0.34 respectively which were not significantly different compared to NC group, however, they were significantly ($p<0.05$) lower compared to MSC and ALA groups. The representative liver sections of MF treated rats showed marked improvement in steatotic changes and hepatocyte ballooning with the disappearance of the interlobular inflammation and almost normal morphological appearance; moreover, there was an absence of collagen around the central vein and in between hepatocytes (Table 5, Figure 2).

Discussion

The current study revealed that treatment with ALA (10 mg/kg/day) lowered the elevated SBP, mitigated the insulin resistance, improved the deteriorated lipid profile and liver enzymes, inhibited the hepatic IL-17 production, increased hepatic adiponectin as well as it lowered the hepatic leptin levels and serum AGEs, and improved liver histological derangements worsened by HCFD feeding. The effects of ALA were compared to those of MF, used as a reference drug, which showed better results in all studied parameters except AGEs. These current findings seem relevant to the management of patients with MS since improvement of the intercalated features of MS and targeting the disease pathogenesis especially the high AGEs levels are considered an important pharmacotherapy targets in order to prevent the high incidence of morbidity and mortality of MS, the worldwide health threat, and to add a new tool for management of this disease.

In the present work, ALA treatment did not decrease BW, LW and LW/BW% ratio. These findings are in line with Park *et al*[16] who reported that ALA did not affect BW of

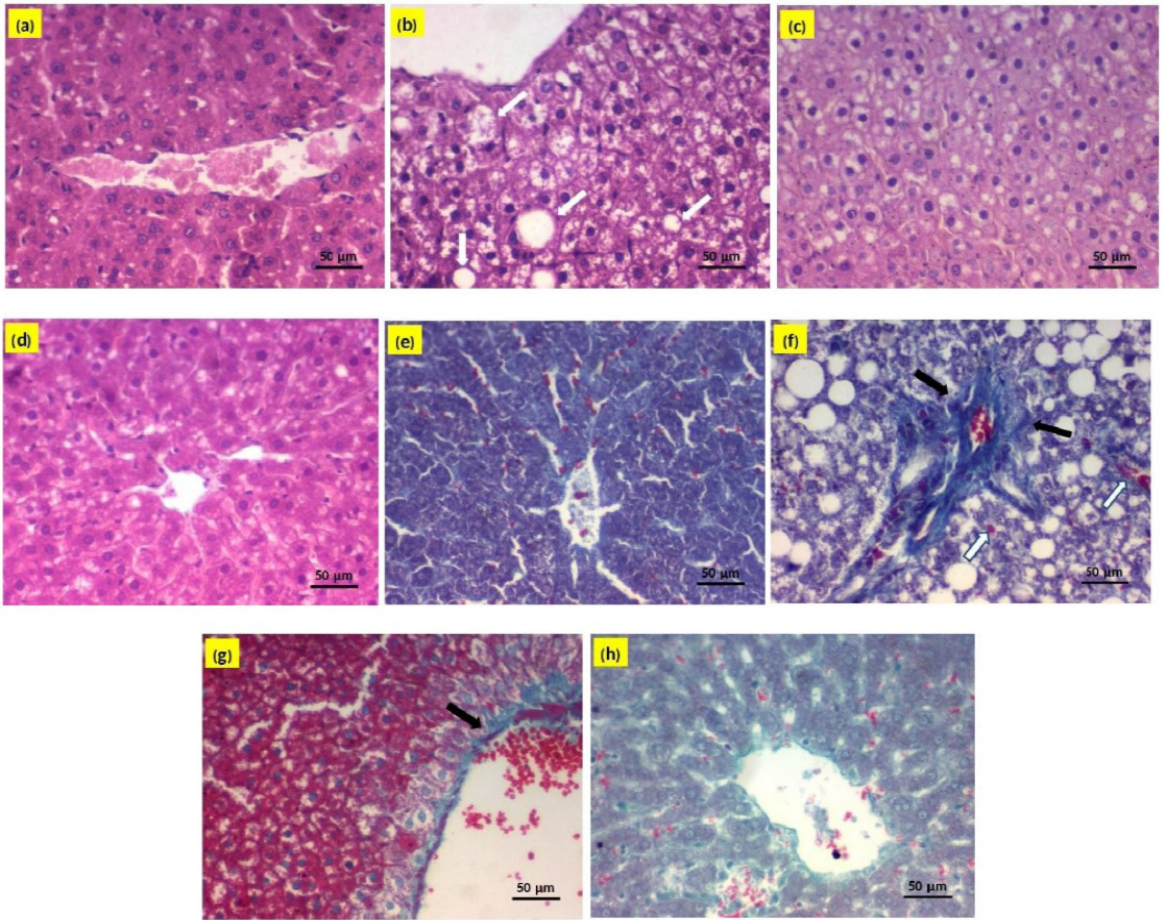

Figure 2. Effect of ALA and MF treatment on hepatic histopathological sections.
(a) liver section of NC group H&E ×400 (scale bar= 50 µm) showing normal sized central vein surrounded by rows and cords of normal hepatocytes with central nuclei and abundant eosinophilic cytoplasm **(b)** liver section of MSC group H&E ×400 (scale bar= 50 µm) showing sever fatty changes of hepatocytes with vaculated cytoplasm (white arrows) **(c)** liver section of ALA group H&E ×400 (scale bar= 50 µm) showing improvements of the fatty changes of the hepatocytes with some vaculated hepatocyte cytoplasm **(d)** liver section of MF treated group H&E ×400 (scale bar= 50 µm) showing marked improvement of the hepatocytes fatty changes **(e)** liver section of NC group MT×400 (scale bar= 50 µm) showing absence of fibrosis around the central vein and in between liver cells **(f)** liver section of MSC group MT ×400 (scale bar= 50 µm) showing excessive deposition of collagen bands (black arrows) with aggregated inflammatory cells (white arrows) **(g)** liver section of ALA group MT×400 (scale bar= 50 µm) revealed moderate collagen bands (black arrow) around the central vein **(h)** liver section of MF treated group MT×400 (scale bar= 50 µm) revealed absence of collagen around central vein and in between hepatocytes, with no inflammatory cells.

Table 5. Effect of single daily oral dose of 10 mg/kg alagebrium, 50 mg/kg metformin for 6 weeks on male Wistar rat liver histopathological features: The histological changes of the liver are according to Nonalcoholic Fatty Liver Disease (NAFLD) Activity Score (NAS) and the fibrosis staging that were performed by using the method described by Kleiner *et al.*[33]

Group (n=8)	Total NAS score	Stage of fibrosis
NC group	0.13 ± 0.35^{A}	0 ± 0.0^{A}
MSC group	7.3 ± 0.91^{B}	1.6 ± 0.51^{B}
ALA group	4 ± 1.51^{C}	1.13 ± 0.35^{C}
MF group	0.5 ± 0.75^{A}	0.13 ± 0.34^{A}

NC: normal control group; **MSC**: metabolic syndrome control group; **ALA**: alagebrium treated group; **MF**: metformin treated group **NAS:** Non-alcoholic fatty liver disease Activity Score; **n**: number of rats in each group. Statistical analysis was done by Kruskal Wallis test followed by Mann-Whitney's test. Values represent (Mean ± SD). Values in the same column with different superscript capital letters are significantly ($p<0.05$) different compared to each studied group.

diabetic mice. In contrast, Harcourt *et al*[34] found that ALA significantly reduced epididymal and omental adipose tissue deposition and the BW of obese mice. The author explained their finding to the lowering effect of ALA on AGEs that overproduced in obesity and its ability to interfere with the AGE/RAGE (receptor of AGE) axis. This controversy may be due to the use of different animal species used in the present study. However, MF reduced the BW, LW and LW/BW% ratio, these results agree with El-Lakkany *et al*[35] study in rats fed high-fat diet. The effect of MF in counteracting adipose tissue expansion was attributed to its anorexigenic effect as mentioned by Kim *et al*[36] however, Tokubuchi *et al*[37] mentioned that by facilitation of fat oxidation MF beneficially reduced visceral fat volume independent of its anorexigenic effects. ALA treatment significantly reduced SBP in the current work which is consistent with Zhang *et al*[38] who found that SBP significantly decreased after use of ALA on diabetic-

hypertensive rats, and they attributed the antihypertensive effect of ALA to the indirect increase in nitric oxide (NO) and prostacyclin (PGI_2) as ALA reduces AGEs accumulation in blood vessels, thereby benefiting the synthesis or secretion of NO and PGI_2 by vascular endothelial cells. However, Toprak *et al*[17] mentioned that blockage of L-type calcium channels explains the vasodilator effect of ALA. MF treatment also reduced SBP and this finding is in line with Shishavan et al[39] who reported a significant reduction in the elevated blood pressure in diabetic hypertensive rats by MF. The antihypertensive action of MF was attributed to the increase in NO bioactivity that improves the endothelial vascular function after MF treatment.[39] Moreover, the ability of MF to reduce vascular production of vasoconstrictor PGs, that occurs secondary to the reduction in hyperglycemia[39]could be another explanation of the antihypertensive mechanism of MF as mentioned by Puyó *et al.*[40]

Regarding FBG in the current work, ALA lowered their levels which agree with Harcourt *et al*[34] study in obese mice. The contribution of these present findings was clarified by Singh et al[41] who mentioned that AGEs are considered an important risk factor in the development of insulin resistance and the impairment of the insulin-mediated metabolism of glucose and so the elevated plasma glucose levels and the development of the diabetic complications. Moreover, Fujimoto *et al*[42] stated that ALA could reduce the levels of AGEs, the same finding was observed n the current work, through breaking their cross-links and so reversing their deleterious effects on glucose homeostasis and other metabolic disturbances.

Treatment with MF reduced FBG levels, these findings are in accordance with Meng *et al*[43] who showed that MF reduced the serum blood glucose levels and improved the glucose metabolism in rats with type-2 DM especially when used with high-fat diet rather than the standard diet. The author explained the improvement of hyperglycemia with MF to the suppression of glucose production by the liver and the stimulation of glucose uptake in muscle and adipose tissue.

Concerning serum insulin levels and HOMA-IR in the present study, ALA significantly reduced their levels that agree with Dhar *et al*[44] study on Sprague-Dawley rats. Such a finding could explain the improvement of insulin resistance after ALA treatment. MF also significantly reduced serum insulin levels and HOMA-IR. Which are in line with Al-Rasheed *et al*[45] who found that MF decrease serum insulin levels and attenuated insulin resistance in rats with MS attributing their finding to the improvement in the impaired insulin signaling which also proved in the present study by increasing the pAkt/Akt ratio.

Treatment with ALA improved the lipid profile, as it reduced serum TGs and total cholesterol levels. These results are in agreement with the study of Watson *et al.*[46] The improvement in the serum lipid profile with ALA is attributed to the reduction in the AGEs levels which breaks their cross-links as mentioned by Fujimoto et al[42] which was also proved in the current work. MF treatment also reduced TG and total cholesterol which agrees with Li et al,[47] attributing the lipid-lowering actions of MF to its important effects on bile acids, known to have lipid-lowing effects, which leads to improvement in glucose as well as lipid metabolism by their action on farnesoid X receptor (FXR).

Regarding liver enzymes, treatment with ALA showed an improvement in serum levels of liver enzymes which agree with Fernando *et al*[25] who attributed the improvement in liver function to the inhibition the activated AGE/RAGE pathway in NAFLD that was initiated by the elevated AGE content in the diet enriched in fat and carbohydrate. MF also improved the abnormalities in liver enzymes and these results are in line with El-Lakkany *et al.*[35] The effect of MF on ameliorating liver enzymes might account for the marked improvement of the liver histopathology and less fatty infiltration in hepatocytes as observed in the histopathological results of the current study.

The hepatic inflammatory cytokine IL-17, involved in exacerbating diet-induced obesity and adipose tissue deposition as well as MS pathogenesis, was decreased after ALA treatment. This anti-inflammatory property of ALA is in line with Coughlan *et al*[26] who mentioned that ALA could reduce the inflammatory cytokine production by breaking the AGEs cross-linkage and disabling the interaction between AGEs and their receptors. In the current work, MF also reduced IL-17 levels. These findings are in line with Araújo *et al*[48] who noticed that MF significantly reduced concentrations of IL-1β, with increased AMPK expression in rats.

In the present work treatment with ALA modulate the levels of adipokines as there it showed significantly lower hepatic leptin and significantly increased hepatic adiponectin levels. To our knowledge, no previous studies investigated this effect. This can be explained by the AGE lowering effect of ALA as mentioned by Jia *et al.*[49] However, the underlying mechanisms of this effect of ALA need further investigation. Also, MF reduced hepatic leptin and increased hepatic adiponectin levels which are in line with the work of Al-Rasheed *et al.*[45] It was mentioned before in the present study that MF lowered the serum insulin level and HOMA-IR as well as enhanced insulin signaling illustrated by the increase of hepatic P-Akt/Akt ratio, the observed increase in adiponectin and decrease in leptin with MF treatment exerts insulin-sensitizing and anti-inflammatory actions in liver.

In the present study, ALA treatment increased the hepatic p-Akt/Akt ratio. To our knowledge, there is no study investigated this effect. The improvement in the insulin receptor signaling observed in the present study due to an increase in the hepatic ratio of p-Akt/Akt after ALA treatment may be attributed to its breaking action to the cross-links of AGEs, besides its anti-inflammatory effect proved by the decrease in IL-17 as described before. It was reported that the AGEs produced in diabetes and MS may contribute to resistance of insulin hormone by different

mechanisms, including production of TNF-α, modification of the insulin molecule directly leading to impairment of its action, generation of oxidative stress, and impairment of mitochondrial function, and activation of their cellular receptors.[50]

Histopathological sections of rats received ALA was significantly improved. Fernando *et al*[25] reported similar findings. The author mentioned that the ability of ALA to scavenge the advanced lipoxidation end products (ALEs) that result from the action of reactive oxygen species on polyunsaturated lipids, which claimed to be the cause of fatty liver, is due to its ALEs cross-linkage breaking action. The improvement in hepatic tissue pathology is also consistent with the ameliorating effect of ALA on serum liver enzymes and other biochemical results. The hepatic architecture of liver sections of MF treated rats was also improved. Similar findings are reported with El-Lakkany *et al*[35] who observed markedly attenuated steatosis, inflammation and hepatocytes vacuolation in rats treated with MF.

Conclusion

Alagebrium (10 mg/kg/day) showed a protective role in mitigating MS disturbances evidenced by improved metabolic parameters, amelioration of insulin resistance, and reduction of IL-17 inflammatory cytokine with improvement in the histopathological derangements. The underlying protective mechanism could be due to its ability to lower hepatic AGEs which are implicated in the disease pathogenesis and progression. In conclusion, ALA is a promising agent in the treatment of MS.

Ethical Issues

The study protocol was approved by the Institutional Review Board and ethical committee at Zagazig University, Faculty of Medicine, Egypt (ZU-IRB# 3521) and also the National Institutes of Health Guide for care and use of laboratory animals (NIH Publications "No. 8023, revised 1996") were followed.

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