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Ameliorative Effects of Moringa oleifera Leaf Extract on Mitochondrial Dysfunction, Oxidative Stress, and Insulin Resistance in High-Fat Diet-Induced Metabolic Syndrome in Rats

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ABSTRACT:

Background: Metabolic syndrome includes a combination of conditions, which are dyslipidemia, frequent hyperglycemia, obesity, elevated HOMA-IR, oxidative stress, inflammation, and dysregulation of mitochondrial function. Many bioactive components in Moringa oleifera can promote the balance of metabolism; however, the impact of Moringa oleifera on the pathways of mitochondrial biogenesis is uncertain.

Aim: This study aims to investigate the effects of Moringa oleifera ethanolic leaf extract on mitochondrial biogenesis, inflammation, oxidative stress, mitochondrial dysfunction, and insulin resistance, as well as the expression of genes related to mitochondrial biogenesis, in rats with metabolic syndrome.

Methods: Forty male Wistar rats were randomly assigned to four groups of 10. One group was the negative control; one group was the metabolically disrupted control; one group was treated with metformin; and one group was treated with Moringa oleifera extract. Induction of disruption of metabolism by diet (high fat) was for 8 weeks. Treatments for the remaining 8 weeks by gavage were 200 mg/kg/day metformin and 300 mg/kg/day of Moringa oleifera leaf extract. The following were measured and compared: weight, glucose levels, insulin levels, HOMA, fasting levels, lipids, inflammatory response, oxidative stress,

Natural Resources for Human Health
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liver and kidney function, ATP levels, mitochondrial potential, and ROS levels. Expression levels of SIRT1, PPARGC1A, NRF1, and TFAM were also measured.

Results: Compared to the negative control group, the metabolically disrupted group that did not receive treatment (control for metabolic disruption) showed a statistically significant elevated weight, levels of fasting glucose, total and HOMA insulin, lipids and levels of biomarkers for hepatic and renal function, inflammatory cytokines, MDA and mitochondrial reactive oxygen species, and a decrease in the activity of antioxidant enzymes, ATP levels, mitochondrial potential and significantly decreased the expression of SIRT1 and PPARGC1A, NRF1 and TFAM. Compared to the untreated metabolic syndrome group, Moringa oleifera treatment improved various metabolic and inflammatory conditions ($p < 0.001$). However, the contrasting effects of Metformin and Moringa oleifera include improvements in HOMA-IR and in various antioxidant enzymes. HOMA-IR showed a positive correlation with malondialdehyde and mitochondrial ROS, and a negative correlation with ATP, mitochondrial membrane potential, and mitochondrial gene expression.

Conclusion: Methanolic extract of Moringa oleifera leaves has the potential to serve as a therapeutic agent for treating Metabolic Syndrome, as demonstrated by its effects on several components of metabolic syndrome, including the oxidative and inflammatory challenges associated with mitochondrial bioenergetics and biogenesis. However, more clinical and mechanistic studies are necessary to validate these therapeutic effects of Moringa oleifera.

INTRODUCTION

Obesity is considered a chronic metabolic disorder that involves an excessive accumulation of body fat and is one of the main contributors to type 2 diabetes mellitus, insulin resistance, dyslipidemia, hypertension, and metabolic syndrome. Fat tissue actively participates in metabolism and is more than just an energy storage depot. Obesity leads to excessive tissue inflammation and homeostatic dysregulation by disrupting insulin and glucose metabolism. Obesity casts the largest shadow of all risk factors on insulin resistance and metabolic syndrome (Fahed et al., 2022).

The metabolic syndrome is a collection of abdominal obesity, insulin resistance, high blood pressure, low levels of high-density lipoprotein cholesterol, and high levels of triglycerides. This condition results from high levels of inflammation and oxidative stress, imbalanced hormones and adipokines, and disruptions in energy metabolism. An abundant high-fat diet results in very high levels of free fatty acids and leads to fat deposition in the liver and other metabolically active tissues. Insulin's ability to aid in glucose uptake is negated, and insulin's hepatotropic effects are augmented. These conditions are the most favorable for testing potential therapies for metabolic syndrome and for understanding its pathobiology (López et al., 2018).

An abundance of nutrients promotes the production of reactive oxygen species (ROS) due to leaking electrons at the mitochondria, lipid peroxidation, activation of NADPH oxidase, and the activity of inflammatory cells. If the production of ROS surpasses the capability of the body's intrinsic systems, damage can occur to cellular proteins, lipids, mitochondria, and nucleic acids. This oxidative imbalance can be assessed by measuring levels of lipid peroxidation products, such as malondialdehyde, and the status of antioxidant systems, including glutathione, superoxide dismutase, catalase, and glutathione peroxidase. It has been shown previously that obesity induced by a high-fat diet in rats increases oxidative stress and disrupts metabolic hormones (Othman et al., 2019).

Increased oxidative stress (OS) activates certain stress-sensitive pathways. OS, along with mitochondrial dysfunction (MD), can inhibit the insulin receptor substrate (IRS) pathways. In the presence of these parameters, glucose transporter translocation to the cells is diminished. This state increases blood glucose levels by increasing hepatic gluconeogenesis. As diabetes progresses, especially in the insulin-resistant/obese state, there is compensatory hyperinsulinemia, increased fasting glucose levels, and an increased homeostatic model assessment of insulin resistance index. In this context, it has been reported that *Moringa oleifera* has

positive effects on the insulin-sensitive Akt pathway and on glucose regulation in diet-induced obesity. This leads to the hypothesis that *Moringa oleifera* may have beneficial effects on obesity by restoring certain intracellular insulin signaling pathways (Attakpa et al., 2017).

Although the presence of insulin resistance and metabolic syndrome requires the continuation of conventional drug therapy, there is a growing interest in the use of medicinal plants that have an important biobarrier, such as *Moringa oleifera* Lam. This contains numerous metabolites, including isothiocyanates, phytosterols, phenolic compounds, flavonoids, and alkaloids, and also exhibits lipidemic, glycemic, and oxidative stress and inflammation-control activities. Among the various parts of *Moringa oleifera* Lam., leaves are the most dominant in research and are easily processed into aqueous and ethanolic extracts. There are experimental and clinical studies indicating that supplementation with Moringa oleifera improves metabolism and the regulation of glucose homeostasis and lipids, and reduces inflammation and oxidative stress. All of this provides a scientific rationale for preventing metabolic syndrome with *Moringa oleifera* (Adarthaiya & Sehgal, 2024).

Interest in *Moringa oleifera* as an anti-obesity and cardiometabolic disorder treatment has increased. The presence of several secondary metabolites among the leaf constituents, such as flavonoids and alkaloids, suggests the potential of Moringa oleifera to act through various complementary metabolic pathways. These constituents have been shown to regulate lipid and glucose metabolism, control inflammation, boost endogenous antioxidants, and protect metabolically active tissues. These broad effects may be due to the involvement of several classes of phytochemicals that enhance the balance of oxidation and metabolism within cells, modulate hepatic lipid metabolism, and improve adipose tissue function and insulin sensitivity (Popoola et al., 2020).

Based on the currently available evidence, *Moringa oleifera* can also exert anti-obesity effects by regulating pathways that control energy metabolism. In the context of obesity induced by a high-fat diet, it is suggested that the anti-obesity effects of *Moringa oleifera* may be due to the regulation of appetite, oxidation of fatty acids, and hepatic lipid using the receptors of the melanocortin-4 and peroxisome proliferator-activated receptor alpha. It has also been reported that various preparations of *Moringa oleifera* may be able to prevent fat accumulation, enhance the metabolism of glucose, improve dyslipidemia, and prevent lipid accumulation in the liver from high-fat and high-fructose diets.

These studies suggest that the plant acts on multiple metabolic levels by regulating energy intake, enhancing lipid oxidation, reducing ectopic fat accumulation, and increasing peripheral insulin sensitivity (Ezzat et al., 2020; Irfan et al., 2020; Kilany et al., 2020; Muhammad et al., 2020). *Moringa oleifera* has the potential to modify metabolism by acting at multiple cellular levels and possibly by altering the gut microbiome. It has been reported that one of its phenolic glycosides activates AMPK, which is the master regulator of cellular and mitochondrial metabolism. The gut microbiome is also a crucial factor in integrating several of the aforementioned mechanisms. Crude polysaccharides from *Moringa oleifera* leaves inhibited diet-induced obesity in mice by modifying the gut microbiome. These mechanisms are highly relevant because the altered gut microbiome, impaired AMPK, and mitochondrial dysfunction can lead to inflammation, fat accumulation, and insulin resistance. While the preliminary evidence from numerous studies is encouraging, differences in plant processing methods, extraction methods, dosages, and biological models all necessitate the use of metabolically standardized plant extracts to ensure measurable outcomes. Studies on toxicity indicate that it is crucial to test the extract's safety before its metabolic effects are evaluated, especially in the case of potential prolonged use in humans (Bao et al., 2020; de Barros et al., 2022; Huang et al., 2023).

Although many studies outline the metabolic benefits of *Moringa oleifera*, its effects on the molecular regulators of mitochondrial biogenesis in experimental metabolic syndrome, compared with the insulin-sensitizing drug metformin, have been largely neglected. Thus, this study aims to investigate the effects of *Moringa oleifera* leaf extract on metabolic syndrome induced by a high-fat diet in rats. Changes in body weight, fasting glucose, insulin resistance, serum lipids, liver function, oxidative stress, and the balance of antioxidants, along with the relative expression of the SIRT1, PGC-1α, NRF1, and TFAM genes, will be measured. The study will evaluate the therapeutic potential, proposed mechanism, and molecular basis of the plant extract in relation to healthy controls, untreated controls, animals with metabolic syndrome, and animals treated with metformin (El-Shehawi et al., 2021).

Natural Resources for Human Health
eISSN: 2583-1194

MATERIALS AND METHODS

Study Design

The effects of *Moringa oleifera* leaf extract on factors associated with mitochondrial dysfunction, oxidative stress, and insulin resistance, and how these relate to metabolic abnormalities linked to metabolic syndrome in high-fat diet-induced rats, were evaluated in this parallel-group study. Metformin was used as a comparative standard.

The study was conducted over 17 weeks, including one week of acclimatization, eight weeks to induce metabolic syndrome, and eight weeks for treatment. During the treatment period, the rats on a high-fat diet continued to receive the treatment to determine whether the administered treatment would alleviate metabolic abnormalities associated with continual exposure to a high-fat diet.

The rats were assigned to one of four experimental groups. The primary outcomes were assessed using the homeostatic model of assessment for insulin resistance, tissue ATP, and the relative expression of mitochondrial biogenesis genes. Secondary outcomes included changes in body weight, fasting blood glucose, glucose intolerance, dyslipidemia, altered liver function, oxidative stress, inflammation, and changes in tissue histopathology.

This study adhered to the guidelines for the care and use of laboratory animals, and all aspects of the study, including housing, dietary treatment, drug administration, blood sampling, and tissue collection, were performed in accordance with these guidelines.

Every effort was made to minimize animal handling and associated stress. Animals were monitored daily, and the following were noted: pain, severe illness, respiratory distress, lethargy, failure to eat and/or drink, and significant weight loss. Animals in severe distress and those with greater than 20% total body weight loss were assessed by a veterinarian and, if required, were euthanized.

Sample Size Determination

An a priori power analysis was used to determine the sample size for an overall one-way analysis of variance with four independent groups. The desired power of the analysis was set at 80%, with a significance level of 0.05 and a moderate effect size of 0.55.

Based on the criteria outlined above, a minimum sample size of 40 was required. Therefore, 40 adult male Wistar rats were used in the study, with 10 rats assigned to each of the four groups. It was determined that this sample size would be adequate to assess potential differences of biological importance in insulin resistance, oxidative stress biomarkers, and mitochondrial gene expression in the experimental groups (Faul et al., 2007).

Experimental Animals

Forty healthy adult male Wistar rats, 8–10 weeks old, weighing 180–220 grams, were used in the study.

In order to reduce biological variability due to the influence of the estrus cycle and the fluctuating hormones of a female rat's reproductive cycle, only male rats were utilized. A veterinary inspection was completed on all experimental subjects prior to their acceptance. Rats were excluded from randomization if they met a set of criteria, including visible congenital disabilities.

The animals were kept in polypropylene cages, each containing clean wood-shaving bedding, with no more than five rats per cage. The animal room was kept at 22 ± 2 degrees Celsius with a humidity of 50 - 60%, along with a 12-hour light/dark cycle.

The rats were allowed ad libitum access to their diet and drinking water, and the cages were maintained and cleaned regularly. The rats were monitored regularly to ensure they maintained good health and to track activity, grooming, and food consumption, and to observe for signs of treatment-related stress and adverse effects.

All of the rats were allowed to acclimatize for seven days before the onset of the scheduled dietary intervention. During this time, all the rats were provided a standard laboratory diet and drinking water ad libitum.

To minimize stress, the rats were handled similarly daily to reduce the stress associated with weighing, oral gavage, and other manipulations required for the study. At the end of the acclimatization period, baseline body weight, fasting blood glucose, food intake, and general health of the rats were recorded and assessed.

Metformin hydrochloride was purchased from a local pharmacy. Metformin was administered via oral gavage at a dose of 200 mg/kg body weight/day. The dosage was adjusted weekly based on the rat's most recent weight.

Collection and Authentication of *Moringa oleifera* Leaves

The leaves of *Moringa oleifera* Lam. were collected from the local market. Leaves were washed with tap and distilled water to remove impurities. They were shade dried until constant weight at RT in a ventilated hood. Leaves were powdered with a mortar and pestle (Othman et al., 2019; El-Shehawi et al., 2021).

Preparation of *Moringa oleifera* Leaf Ethanolic Extract

Extraction was performed using a hydroethanolic solvent of 70% ethanol and 30% distilled water. A 1:10 (w/v) extraction solvent-to-powdered leaves ratio was used.

The solvent extraction mixture was macerated at room temperature for 72 h with intermittent shaking. The mixture was filtered through muslin cloth and Whatman filter paper. The extraction process was repeated twice for each filtration to ensure maximum recovery of phytoconstituents.

The filtrates were collected and rotary-evaporated to remove the solvent, and the temperature was maintained at 40°C. The concentrate was vacuum-dried to remove moisture and to obtain a stable powdered extract.

The percentage extraction yield was calculated based on the following formula:

Extraction yield (%) = Weight of dried extract ÷ Weight of initial dried plant powder × 100

The dried extract was stored in airtight amber containers at −20°C until use. A new suspension in distilled water or 0.5% CMC was prepared each day prior to oral dosing.

Experimental Diets

Standard Diet

The Normal Control (NC) Group was provided with a standard laboratory rodent diet containing approximately 10% of total energy from fat, 20% from protein, and 70% from carbohydrates. The NC diet contained sufficient vitamins, minerals, and fiber to support growth.

High-Fat Diet

A diet containing approximately 45% of total energy from fat, 20% from protein, and 35% from carbohydrates was used to induce metabolic syndrome. The diet was prepared freshly at regular intervals, and appropriate measures were taken to prevent oxidation and contamination (Moreno-Fernández et al., 2018; Zhang et al., 2020).

Induction of Metabolic Syndrome

After the acclimatization period, 30 rats were fed a high-fat diet for 8 weeks to induce obesity, insulin resistance, dyslipidemia, and other characteristics of metabolic syndrome. The remaining 10 rats continued the standard diet to serve as the normal control group (Zhang et al., 2020).

Confirmation of the development of metabolic syndrome after the induction period was based on measurements of body weight gain, fasting blood glucose, fasting insulin, and serum triglycerides, along with the homeostatic model assessment of insulin resistance.

The high-fat diet induced metabolic syndrome in rats, which exhibited increased body weight, impaired fasting glucose or insulin resistance, and dyslipidemia, compared to normal control animals.

Randomization and Allocation

The high-fat diet induced rats with confirmed metabolic syndrome were randomly assigned to the untreated metabolic syndrome group, the metformin group, or the *Moringa oleifera* group.

Randomization was achieved using a method that generated random numbers on a computer. Identification of experimental animals was performed using a nontoxic tail-marking method or by numbering cage cards. The investigators performing laboratory measurements or the histopathological evaluation were blinded to the assigned groups whenever possible.

Experimental Groups

Forty rats were divided into four groups of ten animals each.

Group I: Negative Control Group

These animals were provided with the standard laboratory diet and distilled water for the entire duration of the experiment. During the experimental period, these animals received the vehicle by mouth once daily for 8 weeks.

Group II: Positive Control Group

These animals were provided with a high-fat diet for the entire duration of the induction and treatment periods. These animals received the treatment vehicle by mouth once daily for 8 weeks and were not provided with any active therapy.

Group III: Metformin-Treated Group

These animals were provided with a high-fat diet and received metformin hydrochloride by mouth at a dose of 200 mg/kg body weight/day for 8 weeks.

Group IV: *Moringa oleifera* Extract-Treated Group

These animals were provided with a high-fat diet and received oral gavage of the ethanolic extract of *Moringa oleifera* leaves at a dose of 300 mg/kg body weight/day for eight weeks (Othman et al., 2019; El-Shehawi et al., 2021).

Treatment Administration

The extracts of *Moringa oleifera*, metformin, and the corresponding vehicle were administered to the animals by oral gavage once daily for each treatment, at approximately the same time each day.

The administered volume was less than 10 mL/kg body weight. The treatment doses were adjusted weekly according to changes in body weight.

All high-fat-diet groups were maintained on their respective high-fat diets throughout the eight-week treatment period, and the negative control group continued on the standard diet.

Body Weight and Intake Monitoring

Body weights of individual rats were recorded at baseline and once per week throughout the study. Weights were measured on a commercial balance and used to determine total body weight gain.

Body weight gain = Final body weight − Initial body weight

Body weight gain, expressed as a percentage, was calculated as follows:

Body weight gain (%) = (Final body weight − Initial body weight)/ Initial body weight × 100

Food consumption was estimated based on the weight of food provided to the cage and the weight of food remaining after 24 hours. The daily food intake for each rat was estimated based on the total food weight minus the remaining food weight divided by the number of rats in the cage. The caloric intake was calculated based on the weight of food consumed and the caloric value of the diet.

Oral Glucose Tolerance Test

An oral glucose tolerance test was performed at the end of the treatment period. The rats were fasted for 10-12 hours with free access to water.

To establish a baseline, blood was drawn via the tail, after which glucose was administered by gavage at 2 g/kg body weight. Blood was drawn at 0, 30, 60, 90, and 120 minutes post administration, and glucose concentration was measured using a commercially available glucometer and an enzymatic assay (Ayala et al., 2010).

Fasting Blood Glucose

Fasting blood glucose levels were measured at baseline, following the induction period, and following the completion of the treatment period. Blood collection was conducted following a period of food deprivation of the rats for 10–12 hours overnight.

The glucose oxidase-peroxidase enzymatic colorimetric method was used to determine blood glucose levels following the procedures provided by the manufacturer.

Insulin and Insulin Resistance

For this study, serum insulin concentration was measured using a rat-specific ELISA.

The homeostasis model assessment (HOMA) was used to calculate insulin resistance as follows:

HOMA-IR = Fasting insulin (μIU/mL) × Fasting glucose (mmol/L) ÷ 22.5

If the fasting glucose was measured in mg/dL, it was converted to mmol/L by dividing the value by 18 (Matthews et al., 1985).

Blood and Tissue Collection

At the conclusion of the treatment, the rats were food-deprived for 10–12 hours overnight. Blood was collected via cardiac puncture, and serum was prepared by allowing the blood to clot in a plain tube. Tubes were spun at approximately 3,000 × g for 10–15 minutes. Separated serum was stored at −80° C until analysis.

Post blood collection, the rats were euthanized via exanguination under deep anesthesia. Rapid removal of the pancreas and heart, along with skeletal muscle, adipose tissue of the epididymis, and liver, was conducted following tissue washing with isotonic saline and blotting to remove excess moisture.

The formula for calculating relative organ weight is as follows:

Relative organ weight (%) = Organ weight ÷ Final body weight × 100

After dissection, each organ was separated into multiple segments. One segment was fixed in formalin for routine histopathological analysis. A second segment was preserved in nitrogen and stored at −80°C for future molecular analysis. A third segment was used to measure oxidative stress and the activities of antioxidant and mitochondrial biomarkers after homogenization.

Preparation of Tissue Homogenates

About 100 mg of liver or skeletal muscle was homogenized in ice-cold phosphate-buffered saline or a suitable homogenization buffer at a 1:9 (w/v) ratio.

The crude homogenates were then centrifuged at ~10,000 × g for 15 min at 4°C. The supernatants were collected and stored at −80°C until analysis.

The protein concentration in the supernatants was determined using the Bradford method, so that the tissue biomarkers could be expressed relative to total protein.

Serum Biochemical Measurements

Lipid Profile

Total serum cholesterol and triglycerides, as well as high-density lipoprotein cholesterol, were determined using commercial enzymatic colorimetric kits.

When serum triglyceride concentrations were within the range, low-density lipoprotein cholesterol was calculated using the Friedewald equation as follows:

LDL-C = Total cholesterol − HDL-C − Triglycerides/5

Very-low-density lipoprotein cholesterol was calculated using the equation below:

VLDL-C = Triglycerides/5

The equation to calculate the atherogenic index is as follows:

Atherogenic index = (Total cholesterol − HDL-C) ÷ HDL-C (Friedewald et al., 1972).

Liver-Function Parameters

Serum levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin, total protein, and total bilirubin were measured using commercial colorimetric kits.

Kidney-Function Parameters

A colorimetric measuring method was used in accordance with the manufacturer's instructions to measure serum creatinine, urea, and uric acid.

Oxidative-Stress Biomarkers

Malondialdehyde

Using test kits, malondialdehyde concentration, an indicator of lipid peroxidation, was measured in serum and homogenized tissue by the thiobarbituric acid method. The results were expressed as nmol/mL in serum and nmol/mg protein in tissue (Ohkawa et al., 1979).

Reduced Glutathione

The colorimetric estimation of reduced glutathione involved its reaction with 5,5'-dithiobis (2-nitrobenzoic acid). The results were expressed as mg/dL in serum or μmol/g tissue (Ellman, 1959; Sedlak & Lindsay, 1968).

Superoxide Dismutase Activity

The activity of superoxide dismutase was measured using a test kit that inhibited superoxide-mediated reactions. Results were expressed as units per milligram of tissue protein (Marklund & Marklund, 1974).

Catalase Activity

The activity of catalase was measured by determining the rate of decomposition of hydrogen peroxide. The activity was expressed as units per milligram of tissue protein (Aebi, 1984).

Glutathione Peroxidase Activity

An assay that evaluates the reduction of hydrogen or organic peroxides via glutathione was used to quantify glutathione peroxidase activity. Results are reported as units per milligram of tissue protein (Paglia & Valentine, 1967).

Inflammatory Biomarkers

Concentrations of serum tumor necrosis factor-alpha and interleukin-6 were measured using rat-specific enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' protocols. Concentrations were determined from standard curves and reported in pg/mL. These were assessed to measure the low-grade, chronic systemic inflammatory response associated with obesity and metabolic syndrome (Othman et al., 2019; El-Shehawi et al., 2021).

Biomarkers for Mitochondrial Function

Adenosine Triphosphate

Tissue samples from liver and skeletal muscle for measuring adenosine triphosphate (ATP) levels were evaluated using a luciferase-based bioluminescent assay kit. Tissue samples, either fresh or quickly frozen, were used as per the manufacturer's protocol. A microplate luminometer was used to measure luminescence, and a standard curve was used to calculate ATP. Data are mean values normalized to tissue protein concentration and reported as nmol ATP/mg protein (Lundin, 2000).

Mitochondrial Membrane Potential

Measurement of mitochondrial membrane potential was carried out on isolated mitochondrial tissue using the fluorescent probe JC-1. Measurement of fluorescence intensities of JC-1 aggregates and JC-1 monomers was done at the respective excitation and

emission wavelengths. Mitochondrial membrane potential was expressed as red to green fluorescence intensity ratio (Sivandzade et al., 2019).

Gene-Expression Biomarkers

Four genes involved in mitochondrial biogenesis and in the metabolism of energy were selected.

Sirtuin 1 (SIRT1), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A/PGC-1α), Nuclear respiratory factor 1 (NRF1), and Mitochondrial transcription factor A (TFAM) (Rodgers et al., 2005; Scarpulla, 2008).

SIRT1 was selected for its involvement in sensing cellular energy and in the activation of PGC-1α. PGC-1α was selected as a dominant controller of mitochondrial biogenesis and of the metabolism of oxidative substrates. NRF1 was selected for its role in regulating the transcription of mitochondrial proteins. In contrast, TFAM was selected for its roles in mitochondrial DNA transcription and maintenance.

Approximately 30-50 mg samples of either liver tissue or gastrocnemius skeletal muscle were used to extract total RNA via a provided protocol of either a commercial RNA extraction kit or TRIzol reagent. A spectrophotometer was used to assess the concentration and purity of the extracted total RNA. Samples were accepted if the 260/280 nm absorbance was between 1.8 and 2.1. Extracted RNA was stored at -80 degrees Celsius until cDNA synthesis.

An equal mass of total RNA was converted to cDNA using a commercially available reverse transcription kit. The reaction mixture comprised RNA template, reverse transcriptase, reaction buffer, primers, dNTPs, and nuclease-free water. cDNA synthesis was performed in a thermal cycler, according to the manufacturer's protocol. The synthesized cDNA was stored at -20 degrees Celsius until qRT-PCR analysis.

qRT-PCR was performed using a SYBR Green master mix in an RT-PCR detection system. Each reaction was performed in a final volume of [10 μL] containing SYBR Green master mix, forward and reverse primers, cDNA template, and nuclease-free water. The amplification conditions consisted of an initial denaturation step at 95 degrees Celsius, followed by 40 amplification cycles comprising denaturation, primer annealing, and extension. A melting curve analysis was performed to verify the specificity of the amplified products. The relative expression of SIRT1, PPARGC1, NRF1, and TFAM was quantified in relation to a reference gene, which included glyceraldehyde-3-phosphate dehydrogenase, beta-actin, or hypoxanthine phosphoribosyltransferase 1.

The threshold cycle comparative method was used to assess relative gene expression as follows:

Relative gene expression = $2^{-}\Delta\Delta Ct$

Where:

ΔCt = Ct of the gene of interest − Ct of the gene of reference

And:

$\Delta\Delta Ct$ = ΔCt of the target sample − average ΔCt of the control group (Livak & Schmittgen, 2001).

Statistical Analysis

Data were analyzed using IBM SPSS Statistics software, version (version number), and/or GraphPad Prism software. Continuous data were expressed as means ± standard deviation when normally distributed. The Shapiro-Wilk test was employed, along with histograms and quantile-quantile plots, to assess the data distribution. Levene's test was applied to check the homogeneity of variances. One-way analysis of variance was used to compare means across the four groups. Pairwise comparisons were made using Tukey's honest significant difference post hoc test. For variables measured multiple times, including body weight and fasting blood glucose, the group and time and group-by-time interaction effects were analyzed using repeated-measures analysis of variance and/or a linear mixed-effects model. Correlations of mitochondrial gene expression along with HOMA-IR, oxidative stress biomarkers, and ATP concentration were analyzed using either Pearson's or Spearman's correlation coefficient. Statistical significance was accepted at $p < 0.05$. The exact p-values were reported whenever possible. Along with statistical significance, effect sizes and 95 % confidence intervals were reported.

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RESULTS

Body-Weight Changes

Initially, no statistically significant difference in body weight was observed across the four study groups. After eight weeks of administering a high-fat diet, the positive control, metformin, and *Moringa oleifera* groups all had significantly greater body weights when compared to the negative control group.

The positive control group had the greatest final body weight. Both metformin and *Moringa oleifera* significantly reduced weight gain compared to the untreated positive control group. There was no statistically significant difference between the metformin and *Moringa oleifera* groups.

Table 1. Body weight of the studied groups during the experimental periods

Measurement	Negative control	Positive control	Metformin	*Moringa oleifera*	F-value	p-value	η^2
Baseline body weight (g)	195.92 ± 8.73[a]	200.14 ± 8.39[a]	202.97 ± 7.65[a]	201.46 ± 8.22[a]	1.35	0.273	0.101
Body weight after induction (g)	229.90 ± 9.74[b]	280.19 ± 10.53[a]	283.53 ± 10.51[a]	282.66 ± 11.78[a]	60.11	<0.001	0.834
Final body weight (g)	272.45 ± 9.89[c]	357.71 ± 13.53[a]	301.41 ± 12.77[b]	310.37 ± 17.54[b]	66.70	<0.001	0.848

The analysis of repeated measures indicated that time significantly affected body weight ($\chi^2 = 1099.38$, $p < 0.001$), and the interaction of group and time was also significant ($\chi^2 = 1545.17$, $p < 0.001$). The interaction indicated that the effects of the interventions on body weight changes over time differed significantly.

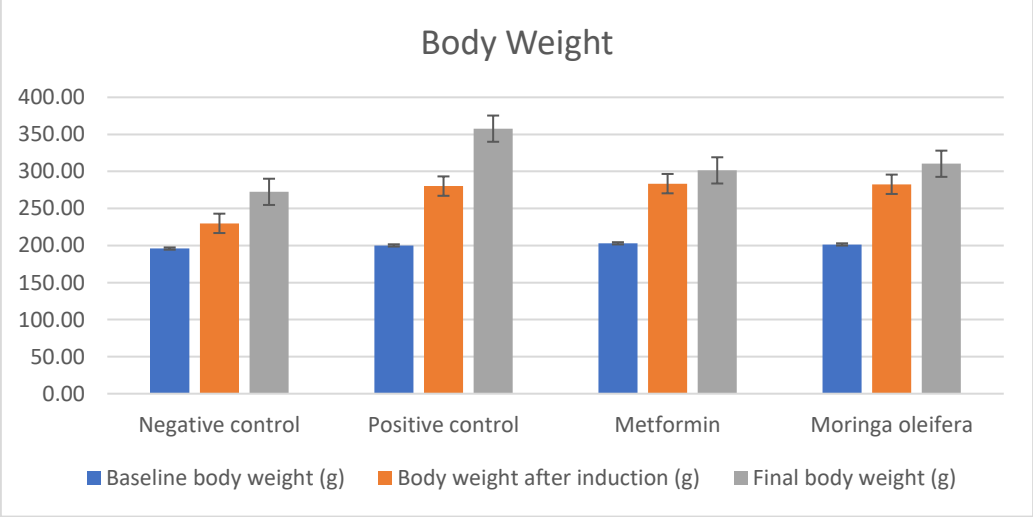

Figure 1. Body weight of the studied groups during the experimental periods

Glycemic Control and Insulin Resistance

The baseline fasting blood glucose levels among the groups did not differ significantly. After the high-fat diet, fasting blood glucose levels increased in all metabolic syndrome groups compared to the negative control group.

After the treatments, the positive control group exhibited significant increases in blood glucose levels, blood insulin levels, insulin resistance, and a decrease in glucose tolerance. Compared to the positive control group, both Metformin and *Moringa oleifera* reduced fasting blood glucose levels, blood insulin levels, HOMA-IR, and the area under the curve for the oral glucose tolerance test.

Compared to *Moringa oleifera*, Metformin produced a significantly lower glucose area under the curve. However, there was no statistically significant difference between the two treated groups in fasting blood glucose, fasting blood insulin, or HOMA-IR.

Table 2. Glycemic parameters and insulin resistance among the studied groups

Parameter	Negative control	Positive control	Metformin	*Moringa oleifera*	F-value	p-value	η^2
Baseline fasting glucose (mg/dL)	90.54 ± 2.44[a]	88.82 ± 1.80[a]	87.82 ± 4.27[a]	88.91 ± 2.77[a]	1.44	0.246	0.107
Fasting glucose after induction (mg/dL)	92.78 ± 9.25[b]	139.23 ± 5.96[a]	140.18 ± 10.31[a]	145.36 ± 7.00[a]	87.28	<0.001	0.879
Final fasting glucose (mg/dL)	92.45 ± 4.37[c]	162.68 ± 14.84[a]	104.82 ± 3.28[b]	112.64 ± 6.95[b]	127.36	<0.001	0.914
Fasting insulin (µIU/mL)	8.54 ± 1.41[c]	25.09 ± 1.87[a]	13.01 ± 1.85[b]	13.88 ± 1.77[b]	164.31	<0.001	0.932
HOMA-IR	1.95 ± 0.34[c]	10.15 ± 1.18[a]	3.56 ± 0.62[b]	4.08 ± 0.66[b]	222.28	<0.001	0.949
OGTT AUC (mg·min/dL)	14,816.91 ± 1,386.55[d]	26,945.19 ± 1,120.18[a]	17,431.26 ± 999.72[c]	19,307.20 ± 733.10[b]	230.84	<0.001	0.951

The assessments of fasting blood glucose levels over time showed significant main effects of time ($\chi^2 = 177.05$, $p < 0.001$) and significant main effects of group by time ($\chi^2 = 549.26$, $p < 0.001$).

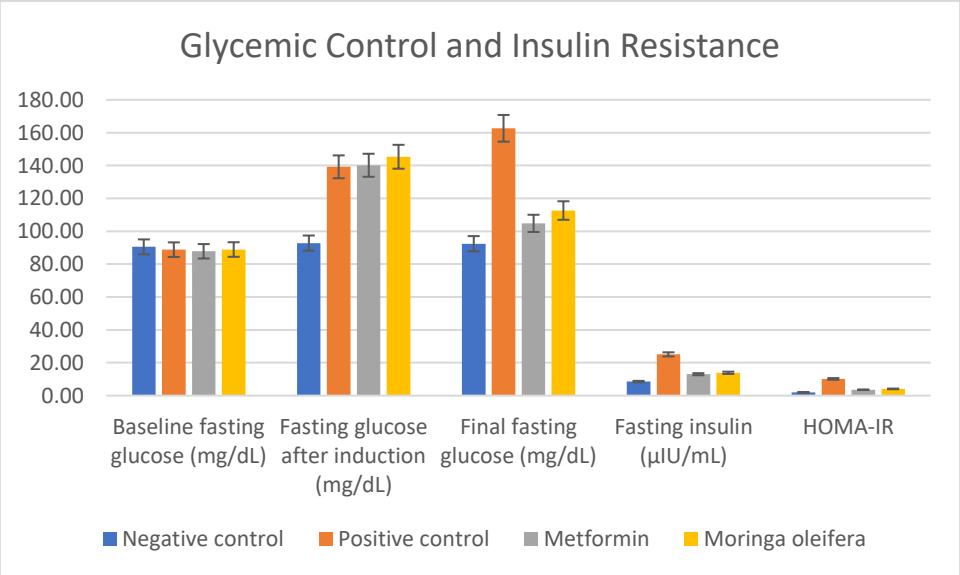

Figure 2. Glycemic parameters and insulin resistance among the studied groups

Serum Lipid Profile

Compared with the negative control group, the positive control group showed pronounced dyslipidemia, characterized by elevated total cholesterol, triglycerides, and LDL-C, and reduced HDL-C.

Compared with the untreated positive control group, both Moringa oleifera and Metformin treatments improved all aspects of the lipid profile. Compared with Moringa oleifera, Metformin treatment had greater effects in reducing total cholesterol and triglycerides and increasing HDL-C. There was no significant difference in LDL-C between the two treatment groups.

Natural Resources for Human Health
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Table 3. Serum lipid profile among the studied groups

Parameter (mg/dL)	Negative control	Positive control	Metformin	*Moringa oleifera*	F-value	p-value	η^2
Total cholesterol	81.36 ± 6.65^{d}	149.05 ± 9.42^{a}	93.55 ± 5.60^{c}	106.15 ± 5.56^{b}	178.01	<0.001	0.937
Triglycerides	67.26 ± 6.17^{d}	157.81 ± 6.96^{a}	89.11 ± 10.49^{c}	99.53 ± 8.78^{b}	218.61	<0.001	0.948
HDL-C	44.79 ± 2.46^{a}	24.85 ± 2.09^{d}	41.42 ± 2.38^{b}	37.50 ± 2.13^{c}	147.45	<0.001	0.925
LDL-C	21.91 ± 9.39^{c}	93.30 ± 6.35^{a}	37.12 ± 6.65^{b}	45.45 ± 4.99^{b}	192.28	<0.001	0.941

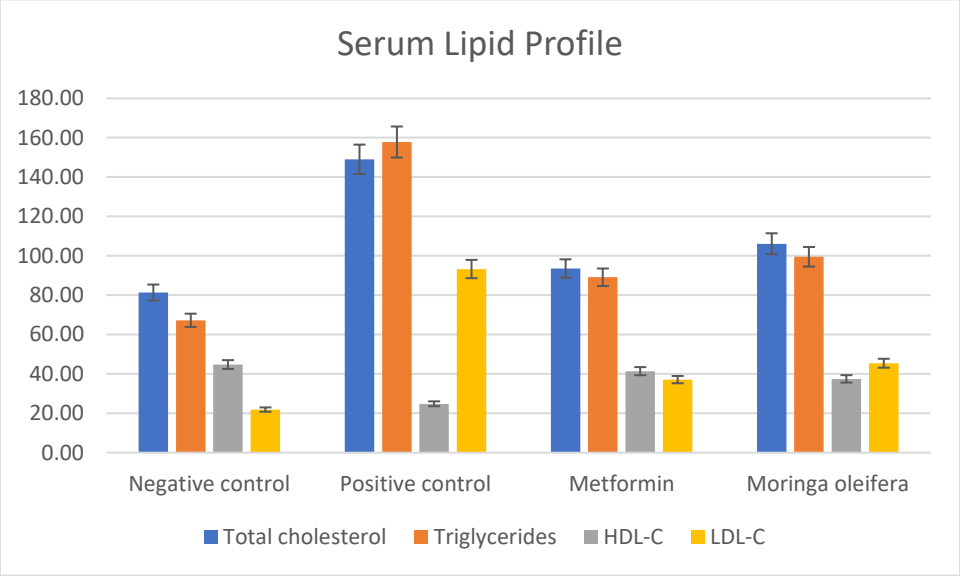

Figure 3. Serum lipid profile among the studied groups

Liver- and Kidney-Function Parameters

Compared with the negative control group, the positive control group showed significant increases in serum levels of ALT, AST, creatinine, and urea, reflecting hepatic and renal perturbations associated with metabolic syndrome.

Compared to the positive control group, the parameters were significantly reduced in both treatment groups. Compared with Moringa oleifera, Metformin treatment significantly decreased ALT levels, whereas AST and urea levels showed no significant differences between the two groups.

Table 4. Liver- and kidney-function parameters among the studied groups

Parameter	Negative control	Positive control	Metformin	*Moringa oleifera*	F-value	p-value	η^2
ALT (U/L)	28.16 ± 3.80^{d}	74.88 ± 5.63^{a}	37.64 ± 5.55^{c}	46.66 ± 5.75^{b}	147.99	<0.001	0.925
AST (U/L)	68.46 ± 4.64^{c}	129.45 ± 10.34^{a}	78.74 ± 8.08^{b}	84.29 ± 6.29^{b}	124.63	<0.001	0.912
Creatinine (mg/dL)	0.65 ± 0.03^{c}	0.90 ± 0.03^{a}	0.69 ± 0.05^{c}	0.74 ± 0.05^{b}	71.16	<0.001	0.856
Urea (mg/dL)	33.01 ± 2.47^{c}	52.36 ± 2.58^{a}	38.05 ± 4.34^{b}	40.76 ± 2.42^{b}	71.84	<0.001	0.857

Natural Resources for Human Health
eISSN: 2583-1194

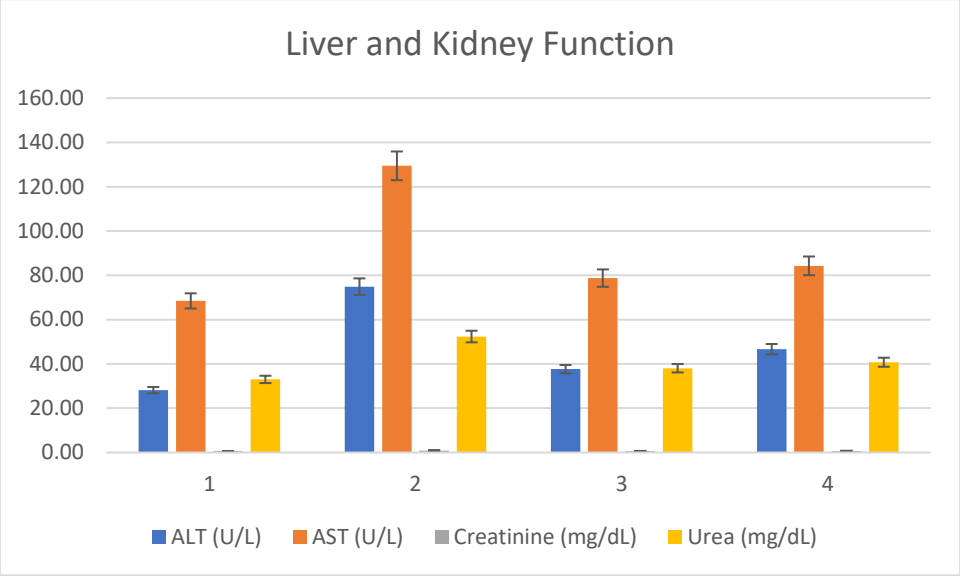

Figure 4. Liver- and kidney-function parameters among the studied groups

Oxidative-Stress and Antioxidant Biomarkers

Compared with the negative control group, the positive control group exhibited increased hepatic malondialdehyde levels and decreased activities of reduced glutathione, superoxide dismutase, catalase, and glutathione peroxidase.

Compared with the positive control group, both metformin and *Moringa oleifera* ameliorated lipid peroxidation and restored the antioxidant defense system. Compared with *Moringa oleifera*, metformin decreased malondialdehyde levels and increased reduced glutathione to a greater extent. There were no significant differences in the activities of SOD, CAT, and GPx between the two treatment groups.

Table 5. Hepatic oxidative stress and antioxidant biomarkers among the studied groups

Parameter	Negative control	Positive control	Metformin	*Moringa oleifera*	F-value	p-value	η^2
MDA (nmol/mg protein)	2.16 ± 0.19^{d}	6.16 ± 0.36^{a}	2.94 ± 0.36^{c}	3.51 ± 0.28^{b}	320.08	<0.001	0.964
GSH (µmol/g tissue)	7.62 ± 0.33^{a}	3.27 ± 0.49^{d}	6.60 ± 0.47^{b}	6.08 ± 0.34^{c}	203.54	<0.001	0.944
SOD (U/mg protein)	48.05 ± 2.38^{a}	25.46 ± 3.40^{c}	42.73 ± 1.83^{b}	39.83 ± 2.43^{b}	140.87	<0.001	0.922
CAT (U/mg protein)	50.98 ± 1.77^{a}	26.75 ± 3.69^{c}	45.95 ± 2.64^{b}	43.41 ± 2.15^{b}	155.30	<0.001	0.928
GPx (U/mg protein)	37.60 ± 1.94^{a}	19.10 ± 3.51^{c}	33.28 ± 2.33^{b}	31.27 ± 1.77^{b}	101.93	<0.001	0.895

Natural Resources for Human Health
eISSN: 2583-1194

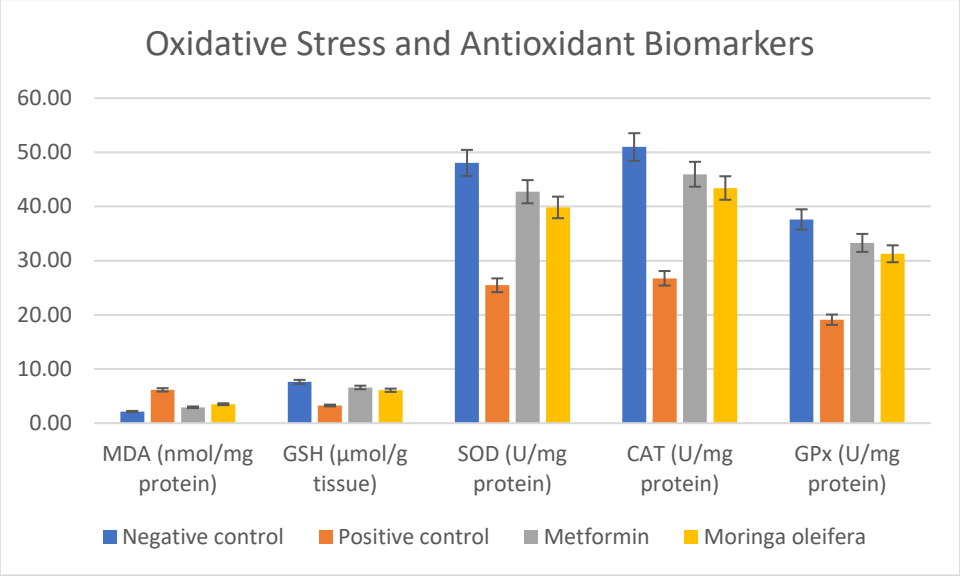

Figure 5. Hepatic oxidative stress and antioxidant biomarkers among the studied groups

Inflammatory and Mitochondrial-Function Biomarkers

Compared to the other groups, the positive control group had much higher serum levels of TNF-α and IL-6. Together, *Moringa oleifera* and metformin lowered these levels, but metformin had a stronger effect.

Compared to the other groups, the positive control group showed major signs of mitochondrial damage: lower levels of tissue ATP, decreased mitochondrial membrane potential, and increased mitochondrial reactive species.

Both *Moringa oleifera* and metformin treatments increased ATP levels, decreased mitochondrial reactive oxygen species, and improved mitochondrial membrane potential. However, metformin had a stronger effect than *Moringa oleifera* on these parameters. There was no significant difference in mitochondrial membrane potential between the two treatment groups.

Table 6. Inflammatory and mitochondrial-function biomarkers among the studied groups

Parameter	**Negative control**	**Positive control**	**Metformin**	***Moringa oleifera***	**F-value**	**p-value**	**η^2**
TNF-α (pg/mL)	25.77 ± 3.29^{d}	66.30 ± 4.44^{a}	34.87 ± 2.87^{c}	39.34 ± 3.98^{b}	222.41	<0.001	0.949
IL-6 (pg/mL)	17.45 ± 2.06^{d}	51.37 ± 4.33^{a}	25.10 ± 3.15^{c}	29.74 ± 2.59^{b}	213.73	<0.001	0.947
ATP (nmol/mg protein)	31.63 ± 1.38^{a}	16.08 ± 2.05^{d}	28.45 ± 1.42^{b}	26.21 ± 1.39^{c}	179.68	<0.001	0.937
Mitochondrial membrane potential, red/green ratio	4.18 ± 0.20^{a}	2.15 ± 0.33^{c}	3.71 ± 0.31^{b}	3.44 ± 0.20^{b}	104.44	<0.001	0.897
Mitochondrial ROS (RFU/mg protein)	105.64 ± 7.89^{d}	227.67 ± 15.89^{a}	126.68 ± 10.62^{c}	151.27 ± 8.15^{b}	229.79	<0.001	0.950

Natural Resources for Human Health
eISSN: 2583-1194

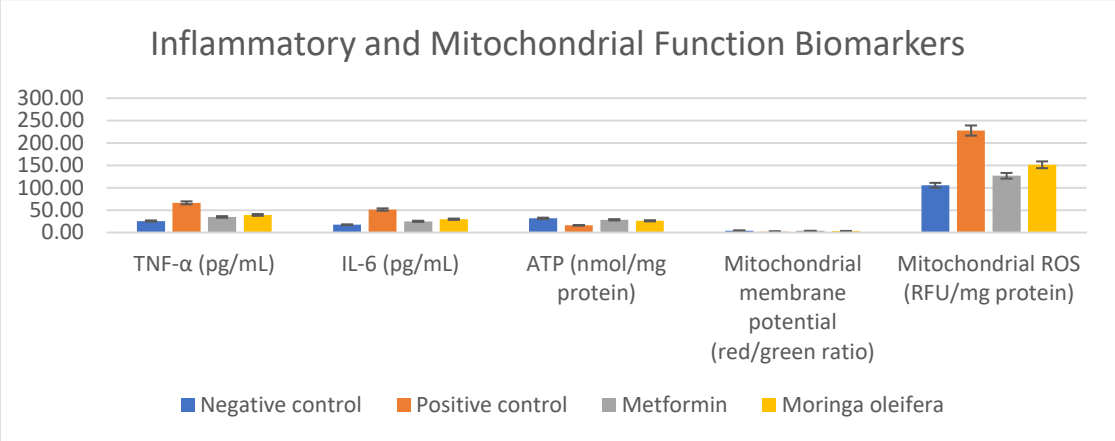

Figure 6. Inflammatory and mitochondrial-function biomarkers among the studied groups

Expression of Mitochondrial Biogenesis-Related Genes

In the positive control group, the expression levels of SIRT1, PPARGC1A/PGC-1α, NRF1, and TFAM were considerably downregulated compared with the negative control group.

Compared to the positive control group, *Moringa oleifera* and metformin treatment increased the expression levels of all four genes. Significantly, metformin treatment led to higher expression levels of all four genes compared with *Moringa oleifera*. However, expression levels of all four genes in both treatment groups were significantly lower compared to the negative control group.

Table 7. Relative expression of mitochondrial biogenesis-related genes in skeletal muscle

Relative gene expression	Negative control	Positive control	Metformin	*Moringa oleifera*	F-value	p-value	η^2
SIRT1	0.99 ± 0.05^{a}	0.44 ± 0.06^{d}	0.86 ± 0.04^{b}	0.76 ± 0.06^{c}	179.33	<0.001	0.937
PPARGC1A/PGC-1α	0.99 ± 0.05^{a}	0.41 ± 0.07^{d}	0.84 ± 0.07^{b}	0.74 ± 0.07^{c}	146.15	<0.001	0.924
NRF1	0.99 ± 0.03^{a}	0.51 ± 0.06^{d}	0.90 ± 0.04^{b}	0.83 ± 0.03^{c}	217.62	<0.001	0.948
TFAM	0.98 ± 0.05^{a}	0.49 ± 0.06^{d}	0.91 ± 0.06^{b}	0.82 ± 0.04^{c}	149.09	<0.001	0.926

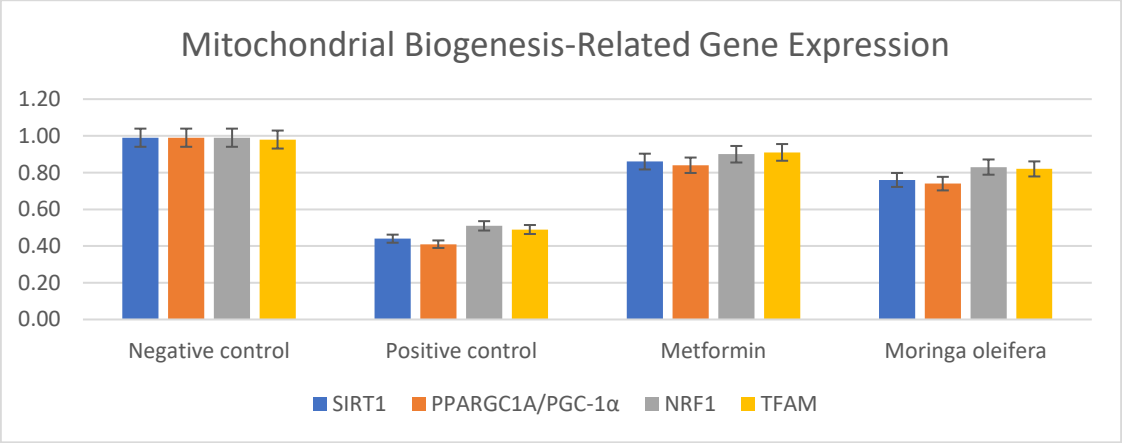

Figure 7. Relative expression of mitochondrial biogenesis-related genes in skeletal muscle

Natural Resources for Human Health
eISSN: 2583-1194

Associations Between Insulin Resistance and Mitochondrial Biomarkers

Correlation with hepatic MDA and mitochondrial ROS was positive for HOMA-IR. Correlation for HOMA-IR with GSH, ATP, mitochondrial membrane potential, and SIRT1, PPARGC1A, NRF1, and TFAM was negative. Insulin resistance was shown to correlate positively with oxidative damage and negatively with energy production in mitochondria and with the expression of the genes associated with mitochondrial biogenesis.

Table 8. Partial correlations between HOMA-IR and selected oxidative and mitochondrial biomarkers

Biomarker	Partial correlation coefficient	95% confidence interval	p-value
MDA	0.730	0.529 to 0.854	<0.001
GSH	−0.639	−0.800 to −0.393	<0.001
ATP	−0.623	−0.790 to −0.370	<0.001
Mitochondrial membrane potential	−0.644	−0.803 to −0.400	<0.001
Mitochondrial ROS	0.650	0.409 to 0.806	<0.001
SIRT1 expression	−0.625	−0.791 to −0.374	<0.001
PPARGC1A/PGC-1α expression	−0.665	−0.815 to −0.430	<0.001
NRF1 expression	−0.619	−0.788 to −0.365	<0.001
TFAM expression	−0.411	−0.651 to −0.095	0.008

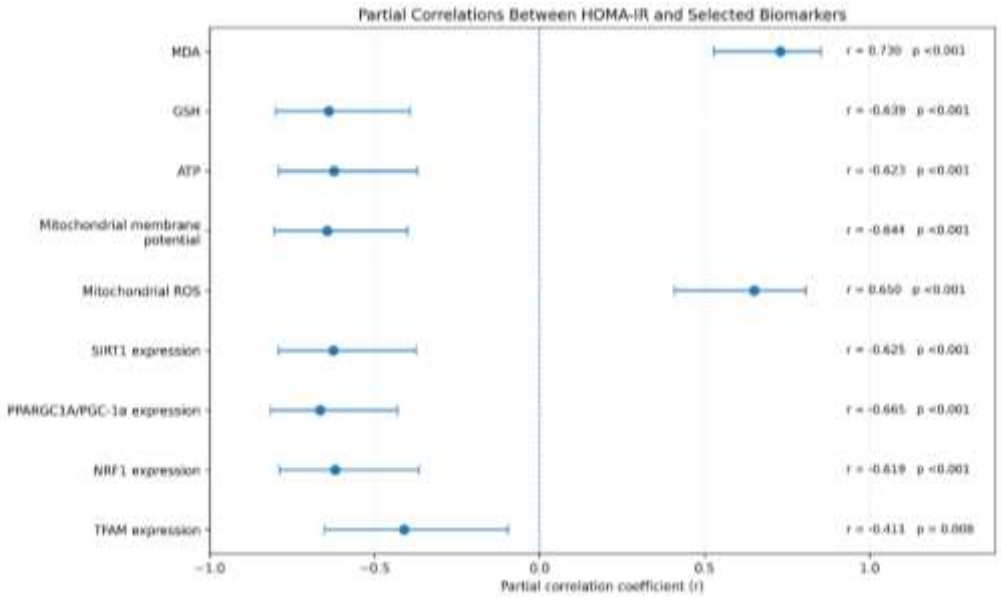

Figure 8. Partial correlations between HOMA-IR and selected oxidative and mitochondrial biomarkers

DISCUSSION

Mitochondrial dysfunction may result from metabolic alterations and be a contributor. With the excess availability of nutrients, we can observe greater than normal loss of electrons from the respiratory chain, generation of reactive oxygen species, lipid peroxidation, and a failure of oxidative phosphorylation. These changes result in reduced ATP production and failure to

produce the components required for mitochondrial growth. This impairs mitochondria's ability to enhance insulin signaling and facilitate glucose utilization. A simultaneous assessment of metabolic, oxidative, inflammatory, and mitochondrial parameters, along with available therapeutic options, yields a more thorough understanding of the effects of Moringa oleifera on metabolic syndrome than a focus on glucose and lipid levels alone.

The objective of this phase of the research was to assess some of the possible effects of the ethanolic extraction of *Moringa oleifera* on insulin resistance, oxidative stress, inflammation, and mitochondrial dysfunction in rats exhibiting diet-induced metabolic syndrome. Forty male Wistar rats were assigned to control and treatment groups, including a metformin control group. Metabolic syndrome was induced by feeding the rats a high-fat diet for 8 weeks, after which treatment was continued for another 8 weeks on the same high-fat diet. Metformin was used as the standard therapeutic comparator.

High-fat diet groups gained weight, with the positive control group showing higher body weight after induction and at the end of the study. The high-fat diet led to an increase in body weight, adipocyte hypertrophy, visceral fat depots, changes in adipokine secretion, and reduced insulin sensitivity, and may have lent the diet a preference for storing fat over other substrates and compromised metabolic flexibility.

Treatment with either metformin or *Moringa oleifera* reduced final body weight significantly compared to the positive control group. Due to the similarity in the final body weights of the two treated groups, it was suggested that the extract had an effect similar to that of metformin. This also agrees with El-Shehawi et al. (2021), in which a dose of 300 mg/kg of ethanolic Moringa oleifera leaf extract significantly reduced body weight gain in rats induced by a high-fat diet. This also agreed with Othman et al. (2019), where *Moringa oleifera* treatment of rats on a high-fat diet also resulted in a significant decrease in body weight gain, and with Mabrouki et al. (2020), where a methanolic leaf extract of *Moringa oleifera* also resulted in significant decreases in changes related to obesity.

The positive control group exhibited marked perturbations in glucose homeostasis, including elevated fasting glucose and insulin, increased HOMA-IR, and an elevated area under the curve on the oral glucose tolerance test. This shows a state of hyperinsulinemia and a marked decrease in insulin sensitivity, which was directly caused by the effect of prolonged exposure to a high-fat diet. High concentrations of free fatty acids in the circulation can disrupt insulin signaling, augment gluconeogenesis, and impede glucose uptake by skeletal muscle, while also causing oxidative and inflammatory stress. The very large effect size for HOMA-IR suggested that the experimental model's greatest impact was the induction of insulin resistance.

Both *Moringa oleifera* and metformin distinctly decreased fasting glucose and insulin levels, HOMA-IR, and glucose AUC when compared with the positive control group. The absence of differences with the fasting glucose, insulin, and HOMA-IR results indicated that the plant extract had a strong effect on insulin sensitization. Our results for *Moringa oleifera* are consistent with those of Othman et al. (2019), who reported improvements in hyperglycemia, hyperinsulinemia, HOMA-IR, and several other metabolic parameters in rats fed a high-fat diet. Attakpa et al. (2017) showed that *Moringa oleifera* decreased blood glucose and insulin levels and reduced weight gain in a diet-induced obesity mouse model by activating the insulin-dependent Akt pathway. As mentioned in El-Shehawi et al. (2021), improvements in serum glucose and metabolic disorders were also reported after administration of the ethanolic leaf extract.

The mechanisms underlying the antidiabetic effect of Moringa oleifera may include potentiation of insulin receptor signaling, increased Akt and insulin signaling, peripheral glucose uptake, inhibition of hepatic gluconeogenesis, and preservation of pancreatic β-cell integrity. The phenolic and isothiocyanate components may inhibit the insulin-induced oxidative stress pathway and stimulate the AMPK pathway. However, when compared with *Moringa oleifera*, metformin had a significantly lower glucose AUC, indicating that *Moringa oleifera* is less effective on the dynamic glucose response. This is likely due to the prevalent mechanisms of metformin, including inhibition of hepatic insulin-dependent gluconeogenesis and modulation of the AMPK pathway.

Current glycemic data are stronger than what is published in the literature. In a randomized study of prediabetes, glycemic control improved with *Moringa oleifera* leaf supplementation, although cardiometabolic parameters showed only limited effects (Gómez-Martínez et al., 2022; Díaz-Prieto et al., 2022).

Marked dyslipidemia, as defined by elevated total cholesterol, triglycerides, and LDL-C, along with a decrease in HDL-C, was observed in the positive control group, and is consistent with the expected results of the high-fat diet model. Dyslipidemia may have been induced by increased triglyceride synthesis, lipid impairment, reduced oxidation, and impaired lipoprotein clearance.

All measured lipid fractions significantly improved with the addition of metformin and *Moringa oleifera*. The study by El-Shehawi et al. (2021) supports the lipid-lowering effects of *Moringa oleifera*. This study also demonstrates that the leaf extract reduced triglycerides, total cholesterol, and LDL-C, while increasing HDL-C in obese rats. In a study by Othman et al. (2019), the extract also reduced the atherogenic index and high-fat-diet-induced dyslipidemia. Improvements in lipid metabolism were also observed in high-fat- and high-fructose-diet models using various preparations of *Moringa oleifera*.

The hypolipidemic effect may be due to several factors, including suppression of cholesterol and fatty acid synthesis, increased oxidation, suppression of lipogenesis, and improved insulin sensitivity. Insulin resistance is associated with enhanced lipolysis in adipose tissue and increased transport of free fatty acids to the liver. The bioactive compounds in *Moringa oleifera* may also activate AMPK and PPAR and modulate the body's preference to utilize lipids rather than store them.

Compared to the plant extract, metformin improved total cholesterol and triglycerides and increased HDL-C. Changes to LDL-C were similar and not statistically different between treatments. These data suggest that *Moringa oleifera* improved the dyslipidemic state, although it does not appear to match the complete lipid-regulating effects of metformin. Strong dyslipidemic effects have been reported in animal models; however, Díaz-Prieto et al. (2022) report no significant changes to the lipid profile in human model prediabetics of 12 weeks of supplementation of 2.4 g/day of dry leaf powder of *Moringa oleifera*. The differences may be related to the type of extract, concentration of extract, metabolic baseline and species differences.

The marked increase in serum ALT and AST in the positive control group indicated high-fat-diet-induced metabolic syndrome along with hepatocellular injury. Increased delivery of free fatty acids to the liver combined with triglyceride accumulation, increased oxidative stress, inflammation, and mitochondrial dysfunction may lead to hepatocyte injury and membrane dysfunction. This would result in leakage of intracellular enzymes to the blood. The increased serum urea and creatinine also likely indicate injury to the kidney.

Moringa oleifera and metformin provided a similar exhibit in alleviating ALT and AST against the positive control group. Othman et al. (2019) reported alterations in ALT and AST, as well as alkaline phosphatase and gamma-glutamyl transferase, due to the effects of *Moringa oleifera* on the high-fat diet. Similarly, El-Shehawi et al. (2021) reported changes in the liver due to biochemical and histopathological effects of the ethanolic leaf extract. Potential mechanisms of the extract may include the inhibition of lipid accumulation, stabilization of the membrane of liver cells, enhancement of the sensitivity of the liver cells to insulin, and inhibition of oxidative stress and inflammation.

The alterations in creatinine and urea may indicate an improvement of the state of the kidney due to the extract. This may have been a result of an improvement in the metabolism of glucose and lipids and a decrease in oxidative stress throughout the body. Creatinine and urea may be indicative of a number of different changes, and the present findings ought to be supplemented by renal histopathology and the other listed biomarkers. It appears that the standard drug provided somewhat stronger protection to the liver than the other treatments. In that regard, the drug decreased ALT to a greater extent than the other treatments; however, there were no significant differences for AST and urea.

There was a significant increase in MDA levels and a decrease in GSH, SOD, CAT, and GPx levels in the livers of the positive control group. These results indicated that high-fat feeding caused severe oxidative stress and reduced the endogenous antioxidant defense system. Increased substrate delivery to the mitochondria results in the formation of superoxide and oxidative stress. Lipid accumulation results in substrate peroxidation. Depletion of GSH combined with reduced antioxidant enzyme activities increases the chance of oxidative stress to the cellular proteins, lipids, and mitochondrial membranes.

Both treatments decreased MDA and increased GSH, SOD, CAT, and GPx compared to the positive control group. These results correlated to the results of Othman et al. (2019), who reported on rats fed a high-fat diet, increased hepatic lipid peroxidation, and reduced levels of GSH, SOD, CAT and GPx, and their restoration after the administration of *Moringa oleifera*. Moreover, El-Shehawi et al. (2021) reported the protective effect of the ethanolic leaf extract on the oxidant-antioxidant balance of obese rats.

The extract also has strong antioxidant potential due to the presence of phenolic acids, flavonoids, and their derivatives. These compounds neutralize free radicals, inhibit lipid peroxidation and reduce prooxidant metals. Metformin decreased MDA levels and increased GSH levels to a greater extent than the other two treatments. There were no differences in SOD, CAT, and GPx levels. Therefore, all treatments were equally effective in restoring the enzymatic antioxidant defense. However, the extract was less effective than the other two treatments in reducing lipid peroxidation.

The positive control group showed significantly increased TNF-α and IL-6 levels due to the presence of obesity-related mild systemic inflammation. Adipose tissue expansion recruits macrophages, alters the secretion of adipokines, and begins the fatty acid-related inflammation pathway, in particular the activation of NF-ϰB. In addition to the elevation of HOMA-IR, it is likely that TNF-α and IL-6 impair insulin receptor signaling.

Moringa oleifera extract also decreased TNF-α and IL-6 levels. This is also observed in El-Shehawi et al. (2021), where *Moringa oleifera* extract inhibited hepatic activation of NF-ϰB and inflammation. Othman et al. (2019) also documented the inhibition of the oxidative and inflammatory disturbance as a major benefit of *Moringa oleifera* extract. Phenolic compounds, isothiocyanates, and other constituents of *Moringa oleifera* may also exert anti-inflammatory effects, since they are capable of the modulation of NF-ϰB, as well as Nrf2 and cytokine production.

Compared to *Moringa oleifera*, Metformin demonstrated a stronger ability to reduce TNF-α and IL-6, likely due to its ability to better mitigate the metabolic-inflammatory response. Additionally, our current findings from animal studies contradicted the findings from the randomized control trial by Díaz-Prieto et al. (2022), wherein participants with prediabetes experienced no significant changes in the inflammatory markers after 12 weeks of supplementation with low dose Moringa leaf powder. The authors proposed that an insufficient dose and variability in baseline inflammatory status may have accounted for the lack of response. Once more, the opposing findings serve as a reminder to take great caution in extrapolating animal studies in which high dose extracts are used to hypothesize human supplement studies.

The positive control group exhibited tissue ATP levels and mitochondrial membrane potential that were significantly diminished, and mitochondrial ROS that were increased, indicating impairment of the mitochondrial electrochemical gradient and leakage with increased electron flow. The decrease in ATP levels and membrane potential indicates important and serious injury to mitochondria and is not merely an increase in the markers of oxidative injury.

Metformin treatment resulted in higher mitochondrial ATP levels and lower mitochondrial ROS than treatment with *Moringa oleifera*. There was no significant difference in mitochondrial membrane potential between the two treatment groups. Therefore, the plant extract likely restored the mitochondrial electrochemical gradient, similarly to metformin. However, it likely offered a less complete restoration of the gradient and a lesser degree of reestablishment of ATP-generating capability and mitigation of electron leakage.

In the positive control group, there was a pronounced downregulation of SIRT1, PPARGC1A/PGC-1α, NRF1, and TFAM, indicating inhibition of the pathway related to mitochondrial biogenesis. SIRT1 is known to activate PGC-1α via deacetylation, and PGC-1α is known to coactivate NRF1 and other transcription factors that are involved in the expression of mitochondrial proteins that are encoded by nuclear genes. NRF1 is known to stimulate the expression of TFAM, which is involved in the transcription, replication, and maintenance of mitochondrial DNA. Inhibition of this pathway likely explains the decrease in both ATP levels and mitochondrial membrane potential.

Both metformin and *Moringa oleifera* significantly upregulated the expression of all four genes when compared with the positive control group. The increased levels of SIRT1 and PGC-1α likely provide a rationale for the restoration of mitochondrial metabolism and improved insulin sensitivity. The increased levels of NRF1 and TFAM likely indicate the stimulation of mitochondrial biogenesis and the enhancement of mitochondrial DNA stability.

Compared to *Moringa oleifera*, Metformin showed a marked increase in gene expression of SIRT1, PPARGC1A, NRF1, and TFAM. Metformin was reported to act on tissues of animals subjected to acute metabolic stress and to stimulate the pathways of mitochondrial biogenesis and increase expression of NRF1 and TFAM. Two treatments were shown to have a greater effect on the above genes when compared to the negative controls. This indicates that the high-fat diet continued to impose a stressor on the animals, thereby sustaining an impairment of mitochondrial function.

The increase in gene expression should be evaluated with caution, as an increase in mRNA does not guarantee an increase in the expression of functional proteins. Stronger evidence of mitochondrial biogenesis could be obtained by the measurement of mitochondrial DNA copy numbers, measurement of the activity of the enzyme citratesynthase, the abundance of the proteins of the electron transport chain, and high resolution respiratory measurements.

HOMA-IR positively correlated with MDA and mitochondrial ROS, while negatively correlating with GSH, ATP, mitochondrial membrane potential, SIRT1, PPARGC1A, NRF1, and TFAM. These associations illustrated that as insulin resistance progressed, oxidative damage increased, and diminished mitochondrial energetic and biogenic potential.

The most significant negative molecular association observed was between HOMA-IR and PPARGC1A, highlighting the key role of PGC-1α in skeletal muscle oxidative metabolism and insulin sensitivity. Insulin signaling may be disrupted due to accumulation of lipid-intermediates from reduced mitochondrial fatty-acid oxidation, which may be attributed to decreased PGC-1α and subsequently reduced mitochondrial biogenesis. The positive associations with MDA and mitochondrial ROS suggested that insulin resistance was compounded by oxidative injury.

These results are in biological agreement with prior studies which show preservation of TFAM and mitochondrial function protects against oxidative stress and insulin resistance induced by a high fat diet. These results are also in agreement with the restoring effect of *Moringa oleifera* on ATP-linked respiration in cells exposed to a high glucose environment.

Correlation does not imply causation. Although the identified relationships remained significant after adjustment for treatment group and final body weight, it is not plausible to infer from these results that either mitochondrial dysfunction was the cause of insulin resistance, the effect of insulin resistance, or if it was part of a bidirectional pathological cycle.

CONCLUSION

The findings demonstrated that constant consumption of high-fat diet leads to obesity, hyperglycemia, hyperinsulinemia, insulin resistance, dyslipidemia, oxidative stress, and inflammation, in addition to some renal and hepatic biochemical disturbances and mitochondrial dysfunction. These changes were associated with the decreased expression of SIRT1/PGC-1α/NRF1/TFAM pathway of mitochondrial biogenesis. On the other hand, the ethanolic extract of *Moringa oleifera* leaves improved body weight, homeostasis of glucose, lipid profile, hepatic and renal functions, inflammation, and oxidative stress, in addition to the production of ATP and the potential of the mitochondrial membrane. It also improved expression of mitochondrial genes and decreased mitochondrial oxidative stress. For several parameters (final body weight, fasting glucose, fasting insulin, HOMA-IR, and LDL-C), the effects of *Moringa oleifera* leaves were similar to those of Metformin. However, Metformin was more effective in the improvement of glucose tolerance, triglycerides, total cholesterol, inflammatory cytokines, ATP, and expression of mitochondrial biogenesis-related genes. The findings indicated that *Moringa oleifera* leaves can exert some protective effects against metabolic syndrome probably due to its combined effects of antioxidant, anti-inflammatory, insulin-sensitizing, and stimulant of mitochondrial biogenesis actions.

Recommendations

Future research should include studies on low, medium, and high dosage treatment groups of *Moringa oleifera* extract in order to determine the minimum effective and optimal therapeutic doses and the dose–response relationship, respectively. Male and female subjects, larger groups, longer studies, and the use of pair-fed groups should be incorporated to determine direct physiological effects of *Moringa oleifera*. Some of the measurements used to substantiate the molecular findings should include protein expression of SIRT1, PGC-1α, NRF1, TFAM, and other parameters involved in the assessment of mitochondrial function and respiratory chain. Combined treatment metformin and *Moringa oleifera* should be studied to determine if there would be any additive or synergistic effects of *Moringa oleifera* on metformin and to study if there are any changes in metformin's pharmacodynamics.

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