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Sedentary Lifestyles and a Hypercaloric Diets During Middle Age, are Binomial Conducive to Fatal Progression, That is Counteracted by the Hormetic Treatment of Exercise, Metformin, and Tert-Butyl Hydroquinone: An Analysis of Female Middle-Aged Rat Liver Mitochondria

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Abstract

The world's population continuous to shift towards older, less active and more sedentary lifestyles especially during middle age. In addition consumption of high-caloric diets, increases the risk of metabolic and cardiovascular afflictions. Developing clinical strategies to mitigate those health complications represent a difficult challenge. Our group has previously shown that combining metformin (MTF) and tert-butyl hydroquinone (tBHQ) treatments, in addition to exercise, partially prevents liver damage associated with obesity. Hence, we evaluated the role of exercise in combination with MTF and tBHQ (triple-treatment) to counteract mitochondrial damage in the liver from obese middle-aged female rats. Animals were fed a high-fat diet (HFD) starting at 21 days till 15 months of age. The treated groups performed a Fartlek-type exercise 5 days/week for 30 min/session. MTF and tBHQ were administered at a dose of 250 mg/kg/day, and 10 mg/kg/day, respectively, for 7 days/month from 10 to 15 months of age. Triple-treatment therapeutic approach promoted animal survival, and increased AMPK and PGC1α expression. Treatments increased mitochondrial ATP synthesis and OXPHOS complexes activities, recovered membrane potential, and decreased ROS production. In summary, exercise in combination with intermittent tBHQ and MTF treatments proved to be an excellent intervention to prevent mitochondrial damage caused by HFD.

Keywords

obesity, female-rats, exercise, mitochondrial respiration, ROS production, ATP synthesis

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Introduction

Scientific and technological advances in disease prevention and treatment have demonstrated profound and significant impact on increasing life expectancy worldwide. According to the Pan American Health Organization report for the year 2022, it was estimated that people aged 60 years at that time could live an additional 20 years, i.e., indicating that of older adults could indeed exceed the 80-year barrier.[1,2] Paradoxically, the World Health Organization[1] (WHO) reported in 2021 that more than 1.9 billion adults worldwide are overweight, 650 million are obese, and about 2.8 million people die each year because of this pandemic. Thus, it has been estimated that the obesity pandemic will lead to increased and significant health care costs as well as decrease life expectancy in many countries.[3] In 2016, Mexico declared obesity has a health emergency, since 72.5% of adults were overweight or obese[4,5]; unfortunately this prevalence has increased in women.[6]

Furthermore, obesity during older age brings with it an additional set of comorbidities that impair the elderly's quality and carries an enormous an economic burden for, the affected individuals their families and society.[7] This situation reinforce the immediate need for better and more effective interventions during middle-age.

At a subcellular level, it has been shown in rodent models and in humans, that high fat diets (HFD) have detrimental effects on mitochondrial homeostasis in multiple tissues. Specifically in the liver of HFD treated rats, transmission electron microscopy has revealed fragmented mitochondria with ultrastructural alterations like swelling, rounding up, and loss of cristae.[8] In regards to mitochondrial bioenergetics evaluated in mitochondria isolated from the livers of HFD-treated Wistar rats, oxidative respiration activity showed a significant decrease when using succinate as a substrate, both in the absence (state 4) or presence of ADP (state 3). These mitochondria also significantly diminished their ATP content.[9] A similar decreased activity in mitochondrial NADH-reductase enzyme (Complex-I) and cytochrome-c-oxidase enzyme (Complex-IV) was observed in high-fat treated diabetic rats).[10] Moreover, García-Berumen and co-workers reported that Wistar rat's liver injury severity, induced by high fructose or high fat diet, is directly associated to the extent of mitochondrial dysfunction, due to reactive oxygen species (ROS) overproduction and deficient oxidative phosphorylation.[11,12] Altered bioenergetics and augmented oxidative stress have also been reported in HFD-treated Sprague Dawley rats, where the mitochondria, besides showing swelling and degeneration, increased the malondialdehyde (MDA) levels and decreased complex I and II, Na^{+}-K^{+}-ATPase, Ca^{2+}-Mg^{2+}-ATPase, superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) levels.[13]

Hence, to try to prevent or reduce the detrimental effects on mitochondria generated by obesity and HFD during aging, a variety of interventions have been developed. One of them that has become very popular is the use of metformin (MTF). MTF is a drug that has been utilized forever than 60 years for the management of type II diabetes.[14] In addition to its therapeutic effects in diabetic patients, MTF has been used in to arrest metabolic dysfunctions, due to its effect in increasing mitochondrial biogenesis,[15] and as anti-hyperglycemic by suppressing hepatic glucose production in an AMPK dependent[16] or -independent ways.[17] In addition to decreasing liver glucose production, MTF also reduces plasma glucose levels by increasing glucose transporter-4 (GLUT4) in skeletal muscle,[18] and is associated with the regulation of glucose metabolism, by inhibition of mitochondrial complex I,[19] activation of AMPK,[20] and suppression of autophagy and inflammation.[21] Two essential aspects of mitochondrial homeostasis are regulated by AMPK: biogenesis and metabolism.[22,23] Under stress conditions, AMPK promotes mitochondrial biogenesis throughout PGC1-α activation, and redirects metabolism towards lipid catabolism and glycolysis by phosphorylating mTOR complex 1 (mTORC1).[24] Interestingly, mitochondrial biogenesis occurs simultaneously by mitochondrial hyper fusion while evading degradation by mitophagy and maximize OXPHOS function.[25] Based on all of these attributes MTF has been proposed as anti-aging instrument.[26]

Another attractive molecule with protective antioxidant effects is tert-butylhydroquinone (tBHQ), which although has not been used in anti-obesity therapy, is a provocative agent as it could protect the mitochondria, and therefore the liver, from fat-induced oxidative damage. TBHQ is a synthetic phenolic antioxidant that protects the cells against ROS-induced damage[27]; it exerts its function by activating the transcription factor Nrf2, which increases several antioxidant enzymes, such as superoxide dismutase 2 and heme oxygenase1 that are known to protect mitochondria during oxidative stress.[28] TBHQ also activates AMPK in hepatocytes, inducing autophagy and protecting them from fatty acid-induced lipotoxicity.[29]

Evidently, the most common non-pharmacological intervention against obesity is exercise. Exercise is known to reduce morbidity and mortality in humans.[30] It does generate a mitochondrial energetic deficit, similar to caloric restriction, which has been recognized as a hormetic mechanism,[31] involving ROS production, mitochondrial unfolded protein response (UPRmt), and release of mitochondria-derived peptides (MDPs) with beneficial metabolic effects.[32] Exercise also has a positive effect on the acceleration of mitochondrial turnover[33] since it increases mitophagy and biogenesis,[34] and it can reduce most of the described hallmarks of aging[33] counteracting age-related decline.[35]

Our group has developed a model to evaluate age-associated osteosarcopenic obesity in female rats, where long-term HFD supplementation was given by feeding them from weaning to 15 months of age.[36] Interestingly, HFD-fed rats did not live longer than that, coinciding with the age at which they can be considered middle-aged animals. In a previous study, a hormetic intervention was performed by

treating HFD-rats with tBHQ and MTF in addition to a Fartlek-type exercise regimen (triple treatment). The pharmacological interventions were administered only 7 days/month from 10 to 15 months of age to obtain a hormetic effect, along with the exercise routine for 5 days a week for 5 months. The triple treatment had a remarkable effect on the rat's survival rate along with the preservation of both mass and muscle strength, body fat loss, and decreased inflammation. Our previous study found that non-exercising HFD rats had a 70% increase in hepatic lipid droplets, and lost liver cytoarchitecture along with inflammatory infiltrates, which improved markedly after the triple treatment. Based on these it was important to understand the effect of exercise in combination with MTF and tBHQ on liver mitochondrial physiology during obesity, with the rationale that the hormetic response could be relevant to explain the beneficial outcomes of the triple treatment. In particular, because the mitochondria play a predominant role in liver physiology and free radicals generation, directly related to inflammation.

The aim of this study was to understand the effects that exercise in combination with MTF and tBHQ on mitochondrial physiology play in the liver of midlife female obese rats. Our hypothesis was that the triple treatment would activate the hormetic response, increasing mitochondrial biogenesis and function. To prove it, hepatic mitochondria were isolated from 15-month-old female Wistar rats subjected to a HFD for 15 months and provided with various treatments, including triple treatment. The expression of AMPK and PGC1α as indicators of mitochondrial turnover, the activity of the electron transport chain (ETC), and the expression of OXPHOS complexes, as well as the mitochondrial membrane potential, ROS production, and ATP synthesis were determined.

Materials and Methods

Animals

Eighty-four Wistar female rats were used in this study. The animals were provided by the closed breeding colony of the Universidad Autónoma Metropolitana-Iztapalapa, México. The rats were kept under standard conditions at room temperature 22 ± 3°C, with an inverted light cycle, 12 hours of light, and 12 hours of darkness, water, and food *at libitum*. All animals were handled according to the criteria established by the National Institutes of Health Guide by the Care and Use of Laboratory Animals, and the Principles of the Mexican Official Ethics Standard 062-ZOO-1999, and the Standard for the Disposal of Biological Waste (NOM-087-ECOL-1995).

Experimental Groups

Eighty-four female rats were used in this study. At 21 days of age, the 84 rats were randomly distributed into the Standard Diet (SD) group (n = 24) and the High Fat Diet (HFD) group (n = 60). At 9 months of age, eight groups were formed from the SD and HFD rats as described by Toledo-Pérez[36] as follows:

(1) *SSD*, Sedentary rats fed with SD;
(2) *SHFD*, Sedentary rats fed with HFD;
(3) *SHFDM*, Sedentary rats fed with HFD and treated with MTF from 10 to 15 months of age;
(4) *SHFDT*, Sedentary rats fed with HFD and treated with tBHQ from 10 to 15 months of age;
(5) *SHFDMT*, Sedentary rats fed with HFD and treated with MTF and tBHQ from 10 to 15 months of age;
(6) *ESD*, rats with Fartlek-type Exercise from 10 to 15 months of age fed with SD;
(7) *EHFD*, rats with Fartlek-type Exercise from 10 to 15 months of age fed with HFD;
(8) *EHFDMT or triple-treatment*, HFD-fed rats treated with MTF and tBHQ along with Fartlek-type Exercise from 10 to 15 months of age.

All groups included 12 animals each, except for the *SHFDM* (n = 6), *SHFDT* (n = 6), and *EHFDMT* (n = 9), due to the fact that not all the rats reached 300 grams of weight (inclusion criteria: less than mean minus two standard deviations from the HFD group). All animals were euthanized at 15 months of age.

One of the limitations of our study is that we could only allow three groups of rats to performed exercise since the number of animals was very large. In addition, we would have liked to compare these results against groups of male rats, but it was not possible to maintain this number of obese male animals. Therefore, we decided to study just female rats, since there is an experimental bias in that most of the studies are performed with males despite the fact many of these diseases and even the pandemic of obesity itself are more pronounced in women.

Animal Diets

HFD was prepared based on an obesogenic diet, consisting of 23.5% protein, 20% lard (40% saturated fat), 5% corn oil (60.7% polyunsaturated fat, 24.3% monounsaturated fat, 15% saturated fat, 0% cholesterol), 20.2% polysaccharides, 20.2% simple sugars, 5% fiber, 5% mineral mix and 1% vitamins (w/w, caloric intake, 4.9 kcal/g).[36,37] The SD groups were fed the Abene BDL-7100 diet, which contained 23% protein, 4.5% fat, and 46.5% carbohydrates (w/w, caloric intake, 3.2 kcal/g).

Exercise Routine

Fartlek type aerobic exercise is a type of practice characterized by increasing and decreasing the displacement speed at different time intervals. The changes of the training load improve physical endurance, aerobic capacity and increase muscular mass.[38] To use this kind of exercise, the animals required an adaptation period to the treadmill (Panlab/Harvard Apparatus) and the routine, which was conducted from the 9th to the 10th

month of age. During that month, the rats were trained to perform the exercise as follows: the first week they were allowed to explore the treadmill for 10 min. The second week they walked at a speed of 12 cm/s for 20 min, which increased to 30 min during the third week. Finally, by the fourth week they walked at 25 cm/s for half an hour. The complete Fartlek-type routine began at the 10th month consisted of 10 minutes of warm-up at low speed (25 cm/s), the next 10 minutes consisted of moderate exercise (50 cm/s) and finally the last 10 min the speed was reduced to 25 cm/s (light exercise). The Fartlek type exercise routine was performed from 10 to 15 months of age; animals were trained 5 days per week for 30 min per session.[36]

Metformin and tBHQ Administration

MTF and tBHQ were used as intermittent treatments to induce a hormetic response. MTF and tBHQ were orally administered at a dose of 250 mg/kg/day[39] and 10 mg/kg/day,[40] respectively, for 1 week (7 days) per month beginning at 10 until 15 months of age.[36]

Liver Mitochondria Isolation

When reaching 15 months of age, the rats were euthanized, and their livers were obtained and washed with SHE-buffer (250 mM sucrose, 10 mM HEPES, and 1 mM EGTA, pH 7.4), fragmented and homogenized. The homogenate was centrifuged at 964 g for 10 min. The supernatant was filtered and centrifuged at 12 000 g for 10 min; the mitochondria were recovered from the pellet and resuspended with a SHE-buffer. The mitochondria sample was centrifuged again at 12 000 g for 10 min. The final pellet was gently resuspended in the SHE-buffer and immediately utilized for mitochondrial assays.[41]

Mitochondrial Respiration

Mitochondrial oxygen uptake was estimated polarographically using a Clark-type electrode (YSI model 5300). Briefly, the mitochondria (1 mg/mL) were resuspended in 1.5 mL of respiration buffer (250 mM sucrose, 10 mM KH_2PO_4, 1 mM EGTA, 10 mM Tris, pH 7.4) with 0.3% BSA. Succinate (10 mM) was used as the respiratory substrate. Stage 3 respiration was initiated with the addition of 100-150 nmol ADP. State 4 respiration was measured as oxygen consumption after ADP consumption. The respiratory control relationship was quantified as the ratio of oxygen uptake at state 3/oxygen uptake at state 4 of the respiration rate.[42]

Mitochondrial Membrane Potential ($\Delta\Psi_m$)

To determine the $\Delta\Psi_m$ the following medium was used: 250 mM sucrose, 10 mM $MgCl_2$, 1 mM EGTA, 0.1% BSA, 10 mM KH_2PO_4, pH = 7.4. Safranin O (5 µM) and 1 mg of mitochondrial protein were added to the spectrophotometer cell. Generation of the $\Delta\Psi_m$ was initiated by adding 10 mM succinate-Tris, pH = 7.4, and 100 nmol ADP was added to membrane depolarization associated with oxygen uptake and ATP synthesis; 5 µM carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) was added to $\Delta\Psi_m$ dissipation at the end of the experiment. The $\Delta\Psi_m$ was evaluated in a double beam spectrophotometer (AMINCO DW 2000, Olis, Inc, Bogart, GA, USA) and the difference of wavelengths was 533-511 nm. The recording was performed in a 3.0 mL cuvette with constant stirring while the temperature was held at 25°C.[43]

Solubilization of Mitochondrial Complexes and Supercomplexes

The isolated mitochondria were solubilized with digitonin to obtain mitochondrial supercomplexes and complexes (ratio of 5 g/g protein) in a solution of 50 mM bis-tris, 500 mM aminocaproic acid, 10 mM succinate, and 10 mM ATP, pH 7.0.[44] The detergent (stock 500 mg/mL in DMSO) was added dropwise, and the solution was incubated for 30 min at 4°C with gentle stirring; subsequently, the mitochondria were ultracentrifuged at 100 000 g for 35 min at 4°C. The respiratory complexes and supercomplexes were recovered in the supernatant. The respiratory complexes (100 µg of protein per well) were resolved by blue native electrophoresis (BN-PAGE) in a linear polyacrylamide gradient gels (4%–10%).[45,46] The anode buffer was 50 mM bis-tris HCl, pH 7.0; the cathode buffer was 50 mM tricine, 15 mM bis-tris, pH 7.0, and the anionic dye Coomassie G250 (0.02%). Electrophoresis was performed at 30 V for 16 h at 4°C and was stopped when the dye line reached the end of the gel. The molecular weight of the respiratory complexes and supercomplexes was determined by their electrophoretic mobility and their in-gel activity (vide infra). Bovine heart mitochondrial complexes solubilized with digitonin under the same conditions were used as molecular weight and activity markers for the gel zymography.[46]

In-Gel Enzyme Activity Assays

Complex I: NADH Dehydrogenase Activity. After the BN-PAGE, the gels were incubated in a solution of 10 mM tris-HCl, pH 7.4, using 1 mM NADH as substrate and 3-[4,5-dimethylthiazol-2-yi]-2,5-diphenyl tetrazolium bromide (MTT) 1.2 mM as an oxidizing agent. The assays were performed at 20-25°C with gentle orbital agitation. After 10-30 min of incubation in the dark, the enzymatic activity in-gel appeared as a purple precipitate; then, the reaction was stopped with the fixing solution (50% methanol, 10% acetic acid).[47]

Complex IV: Cytochrome c Oxidase Activity. The in-gel activity of complex IV was determined using 3,3′-diaminobenzidine

(DAB) as the reducing agent and cytochrome *c* as the electron carrier.[43,44] The gel was incubated in a solution of 10 mM of KH_2PO_4, pH 7.4, DAB 4.7 mM, cytochrome c 4 mg/mL, and 1.8 KU of catalase. The assay was performed at 20-25°C. After 30-40 min of incubation with gentle orbital agitation in the dark, the activity was observed as a brown precipitate. The reaction was stopped by fixing solution.

Complex V: ATPase Activity. The gel location of complex V was determined by its ATP hydrolysis activity. The gel was incubated in a solution of 50 mM glycine, 10 mM $MgCl_2$, 0.2% $Pb(NO_3)_2$, 5 mM ATP, pH 8.0; adjusting the pH with triethanolamine to avoid nonspecific precipitation of lead. ATP hydrolysis activity was observed as a white deposit of lead phosphate, on a dark background. The reaction was stopped with a solution of 40% methanol.[48]

Mitochondrial Electron Transport Chain Complexes Activity

Complex I: NADH Dehydrogenase Activity. Complex I activity was determined spectrophotometrically at 340 nm quantifying the oxidation of 150 µM NADH (ε_{NADH} = 6.22 $mM^{-1}{\cdot}cm^{-1}$) in an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, USA). Mitochondria (10 µg/mL) were permeabilized with 0.5% Triton X-100 and incubated with 30 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, 120 mM KCl, 500 µM dimethoxy-1,4-benzoquinone (DBQ), pH 7.4, as previously reported by.[47] The reaction was started with NADH addition and stopped with rotenone (15 µM); the NADH oxidation sensitive to rotenone was considered as complex I activity. Direct spectrophotometric recording was used for activity quantification.

Complex II: Succinate Dehydrogenase Activity. Complex II activity was determined spectrophotometrically at 600 nm quantifying the reduction of 2,6-dichlorophenol-indophenol (50 µM DCIPIP; ε_{DCPIP} = 21 $mM^{-1}{\cdot}cm^{-1}$). Mitochondria (10 µg/mL) were incubated with 5 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, 20 mM KCl, 200 µM phenazine methosulfate (PMS), pH 7.4, as reported before by De los Ríos-Castillo et al.[49] The reaction was initiated by adding 10 mM succinate. Direct spectrophotometric recording was used for activity quantification.

Complex IV: Cytochrome c Oxidase Activity. Complex IV activity was determined on a Clark electrode, and the mitochondria (1 mg/mL) were resuspended in a respiration buffer (250 mM sucrose, 10 mM KH_2PO_4, 1 mM EGTA, 10 mM Tris, pH 7.4, 540 nM cytochrome c. The reaction was started with the addition of 3 mM ascorbate and 500 nM TMPD and was stopped with 5 mM of KCN as reported before by.[41] The oxygen uptake sensitive to cyanide was considered as CIV activity.

ATP Synthesis by Complex V. Mitochondrial ATP synthesis was quantified in an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, USA) using an assay coupled to $NADP^+$ reduction (ε_{NADPH} = 21 $mM^{-1}{\cdot}cm^{-1}$) as previously reported .[49] The reaction mixture contained 0.5 mM $NADP^+$, 5 mM ADP, 6 units glucose-6-phosphate dehydrogenase/mL, 16 units hexokinase/mL, 10 mM succinate, 100 µM P1, P5-Di (adenosine-5′) Penta-ammonium pentaphosphate, 10 mM glucose, 250 mM sucrose, 20 mM $MgCl_2$, 20 mM KH_2PO_4, pH 7.5. The reaction was started with ADP addition and oligomycin (5 µg/mL) was used to stop the reaction. Direct spectrophotometric recording was used for activity quantification.

ATP Hydrolysis by Complex V. ATP hydrolysis was spectrophotometrically quantified in an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, USA) using an assay coupled to the oxidation of NADH (ε_{340nm} = 6.2 $mM^{-1}{\cdot}cm^{-1}$) as previously reported.[49] The assay medium had 30 mM HEPES, pH 8.0, 6 mM $MgSO_4$, and 90 mM KCl; the ATP regeneration system was 5 mM phosphoenolpyruvate (PEP), 1 mM NADH, 50 units/mL of pyruvate kinase (PK) and 10 units/mL of lactate dehydrogenase (LDH). The reaction was started with the addition of ATP. Oligomycin was used to stop the reaction. Direct spectrophotometric recording was used for activity quantification.

ROS Quantification

The hydrogen peroxide quantification was performed using the Amplex® Red Hydrogen Peroxide Assay Kit (Invitrogen, Molecular Probes, USA). The mitochondria (50 µg/100 µL) were incubated in the buffer included in the commercial kit and supplemented with 10 mM succinate and 3 µM sodium cyanide.[47]

Total Protein Isolation

To isolate the proteins, 25 mg of liver tissue were fragmented and homogenized in lysis buffer (1 mM DDT, 0.1 mM phenylmethylsulfonyl fluoride, 1 tablet of cOmplete™, Mini, Protease Inhibitor Cocktail and 10 mL T-PER). Homogenates were centrifuged at 900g for 10 min at 4°C, the supernatant was recovered.[50]

Western Blot Assays

Thirty µg of liver protein were separated by SDS-PAGE on a 12% polyacrylamide gel under denaturing conditions[51] and then transferred to polyvinylidene difluoride (PVDF) membranes (inMobilon®-P, Millipore bilica, MA), in a semi-dry electroblotting system (Bio-Rad) at 25 V for 50 min. Membranes were incubated at room temperature for 1.5 h in TBS-T (20 mM Tris-HCl, 500 mM NaCl, 0.1% Tween-20) containing 5% skim milk, followed by incubation with the corresponding

primary antibody: PGC1α (1:500; ab106814 Abcam), AMPK (1:500; sc398861 Santa Cruz Biotechnology), or actin (1: 2000; sc47778 Santa Cruz Biotechnology), dissolved in TBS-tween at 4°C. Membranes were washed 3 times with TBS-T for 10 min and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. The same procedure was used for the mitochondrial protein. Membranes were incubated with the primary antibodies Oxphos (1:4000; ab110413 Abcam) and VDAC1 (1:500; sc390996 Santa Cruz Biotechnology), dissolved in TBS-Tween at 4°C. The membranes were then washed 3 times with TBS-T for 10 min and incubated with horseradish peroxidase (HRP) conjugated secondary antibody anti-rabbit or anti-mouse IgG (1: 5000) for 2 h; the proteins were visualized using a chemiluminescent detection system (Millipore, Billerica, MA). The membrane was stripped (0.19 M Glycine, 0.003 M SDS, 2% Tween-20, pH 2.2). The proportion of these proteins was quantified by densitometric analysis, using the Kodak Molecular Imaging software (v.4.5.1).[51] The intensities of proteins were measured by peak integration after densitometry analyses.

Quantification of Protein Concentration

Protein content was determined as described by Lowry et al, [52] with minor modifications[53] BSA was used as standard, and the concentration of the stock solution was quantified by absorbance at 278 nm using an extinction coefficient of 6.58 (1 %).

Statistical Analysis

The data are expressed as the mean ± SEM (standard error of the mean) for at least 3-8 independent experiments per group. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Holm- Sidak´s test for relative expression for OXPHOS complexes, AMPK and PGC1α, supercomplexes, and potential membrane. For the remainder experiments, a Kruskal-Wallis statistical analysis with Dunn´s test was employed. Differences were considered significant when $P < 0.005$.

Results

Exercise and Triple Treatment Increase AMPK and PGC1α Expression

AMPK and PGC1α levels were evaluated as molecules related to cellular energetic level regulation and mitochondrial biogenesis induction (Figure 1A and Supplemental Figure 1). No changes were found in the AMPK levels in the sedentary (S) rats when fed HDF or subjected to any treatment, except for MTF treatment (SHFDM), in which levels were significantly increased (71% vs SSD).

Exercise routine significantly increased AMPK levels in all animal groups. Interestingly, adding MTF and tBHQ to exercise intervention in HFD-fed rats (triple treatment; EHFDMT) induced a significant increase in AMPK levels (more than 2-fold) (Figure 1B). The same behavior was observed for PGC1α, significantly augmented only in those groups who performed an exercise routine (ESD, 69%; EHFD, 129%; EHFDMT, 79%), but not differences were found among them, regardless the treatment (Figure 1C). An increase in AMPK and PGC1α suggests an effect on mitochondrial turnover and biogenesis.

Figure 1D shows the mitochondrial OXPHOS complexes evaluation. No differences were found among the groups, suggesting that the amount of the complexes was not affected by the treatments, but probably their assembly and function were. Hence, their occurrence as free-complexes or supercomplexes, and their functionality were analyzed.

Respiratory OXPHOS Activity as Free-Complexes and Supercomplexes

Figure 2A shows a representative BN-PAGE gel image of the respiratory complexes as free complexes or assembled into supercomplexes (SC). The molecular masses were obtained by using the *Bos taurus* complexes as standard. The bands for complexes I, IV, and V were evidenced by their in-gel activities (Figures 2B-D respectively). The free complex I (free-CI) was associated with the 1000 kDa band, as shown in Figure 2E. The activity of CI as part of the supercomplexes (CI-SC) was associated with several bands of higher molecular weight, especially with the 1560 kDa protein band (Figure 2F).

The relative distribution of CI in its free form or incorporated into the SC was modified in the SHFDM and SHFDT groups (Figure 2B, lane 4 and 5, respectively) with an increase in free-CI activity of 94% and 63%, respectively (Figure 2E), and a decrease of complex I associated with supercomplexes (Figure 2F).

Complex IV (CIV) was mainly found in its free form (200 kDa), while lower activities were associated with different supercomplexes (Figure 2C). It has been reported that CIV could be found to be a dimer (IV_2), associated with complex III_2, (III_2-IV_2) and as part of supercomplexes containing complexes I-III_2-IV. No differences were found in the association of CIV in SC.

Complex V (Figure 2D) was associated with a band of 750 kDa, which corresponds to its monomeric state (CV), while the dimeric form (CV_2) showed a molecular weight of 1500 kDa, but its amount (determined by Coomassie staining) was only minor.

No changes were found in the monomeric state of complex V among the groups; however, V_2 activity decreased by 79% and 75% in the SHFDM and SHFDT groups (Figure 2D, I, and J). Additionally, a band of 300 kDa with ATP hydrolysis was observed in the SHFDM group (Figure 2D, lane 4), which might be the F_1 section of the enzyme. These results confirm

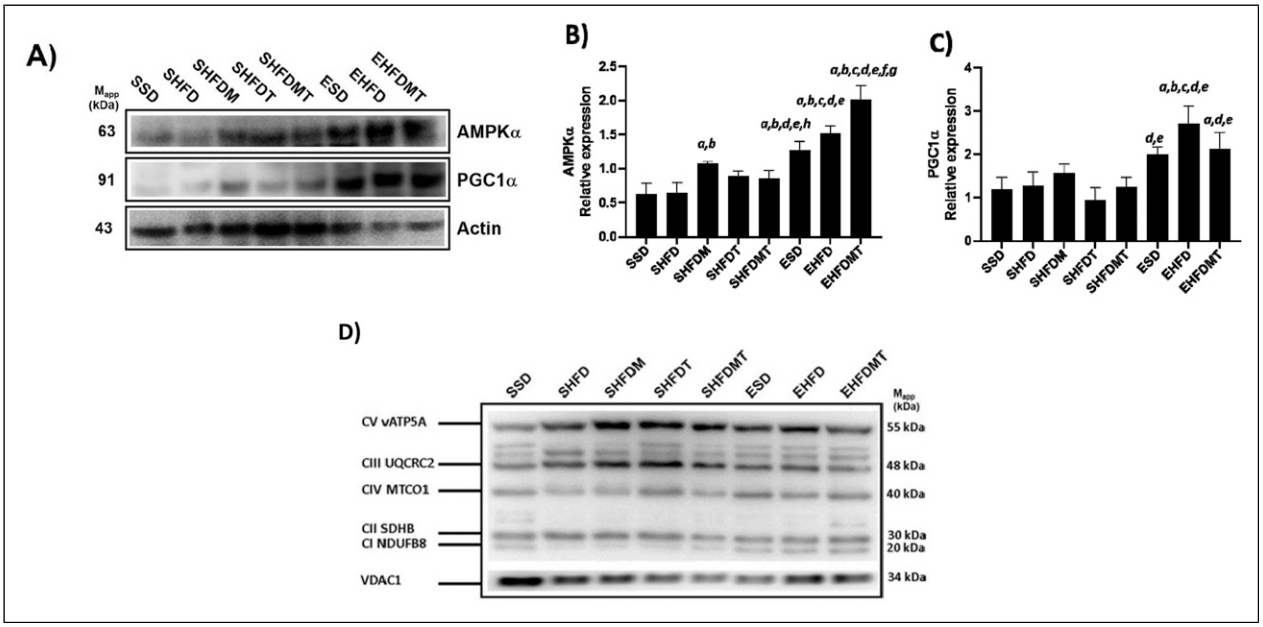

Figure 1. Exercise and triple treatment improve mitochondrial biogenesis in High-Fat-Diet-fed rats. Representative blot of AMPK and PGC1α (A) normalized with actin. Densitometric analysis of AMPK (B) and PGC1α (C). Representative blot showing subunits of OXPHOS complexes (Complex I: NDUFB8; Complex II: SDHB; Complex III: UQCRC2; Complex IV: MTCO1; Complex V: vATP5A and the VDAC1) (D). The comparisons were established using a multivariate test followed by the Holm-Sidak with a $P < 0.05$. The letters represent the significant difference of the groups, i.e. (A) SSD; (B) SHFD; (C) SHFDM; (D) SHFDT; (E) SHFDMT; (F) ESD; (G) EHFD; (H) EHFDMT.

the destabilization of supercomplexes in the SHFDM and SHFDT groups.

Spectrophotometrical Quantification of OXPHOS Activities in Isolated Mitochondria

No differences were observed in the activity of CI in the SHFD, SHFDM, and SHFDT groups compared to the SSD group (Figure 3A), whereas an increase of 50% was observed in the sedentary group with the double treatment (SHFDMT). The exercise group fed with an SD (ESD) showed a decrease of 24% while CI activity increased in the EHFD and EHFDMT groups by 42%, and 97%, respectively (Figure 3A). Complex II (CII) activity in the sedentary groups only decreased in SHFD group by 50% (Figure 3B), while it significantly augmented in the exercised groups, however no differences between them were observed. In the case of Complex IV (CIV) (Figure 3C), all the sedentary groups fed with an HFD decreased their activity regardless of their treatment. The ESD and EHFD groups did not modify their activity in comparison with the control SSD group; while the triple-treatment group (EHFDMT) increased CIV activity by 44%. Regarding the ATPase activity of Complex V (CV), no differences were found in the sedentary groups, except for the SHFDT group, which significantly increased its activity by 30% (Figure 3D). On the contrary, the exercised groups increased their activity, except for the EHFD group. These results show that exercise plus MTF and tBHQ improved the activity of the mitochondrial OXPHOS complexes as compared with the sedentary subjects, regardless of the diet.

Exercise Improves the Mitochondrial Bioenergetic Parameters in HFD Rats

In order to study the effect of the different treatments on mitochondrial bioenergetics, the membrane potential ($\Delta\Psi_m$), oxygen uptake, ATP synthesis, and ROS production, were analyzed.

Mitochondrial respiration was stimulated using succinate as a substrate. No significant differences in the ADP-stimulated state 3 of mitochondrial respiration were found in sedentary groups, regardless of the intervention (Figure 4A). Only in the EHFDMT group increased 1.8 times the oxygen consumption compared with the SSD group (Figure 4A). Regarding state 4 of respiration (i.e. ADP was exhaust), only the SHFD and SHFDMT decreased concerning the SSD, and the triple treatment group increased (EHFDMT) (Figure 4B). The respiratory control ratio (RCR) is an empirical parameter used for assessing the integrity of mitochondria and is defined as oxygen uptake at state 3/oxygen uptake at state 4. Although there is no theoretical value for this parameter, a low RCR value is an indication that mitochondria might be damaged. The HFD actually decreased the RCR in all sedentary groups (Figure 4C), meantime, exercise improved RCR reaching similar values to that of the SSD group. Despite an observed tendency for sedentary or exercise groups, no statistical differences were obtained.

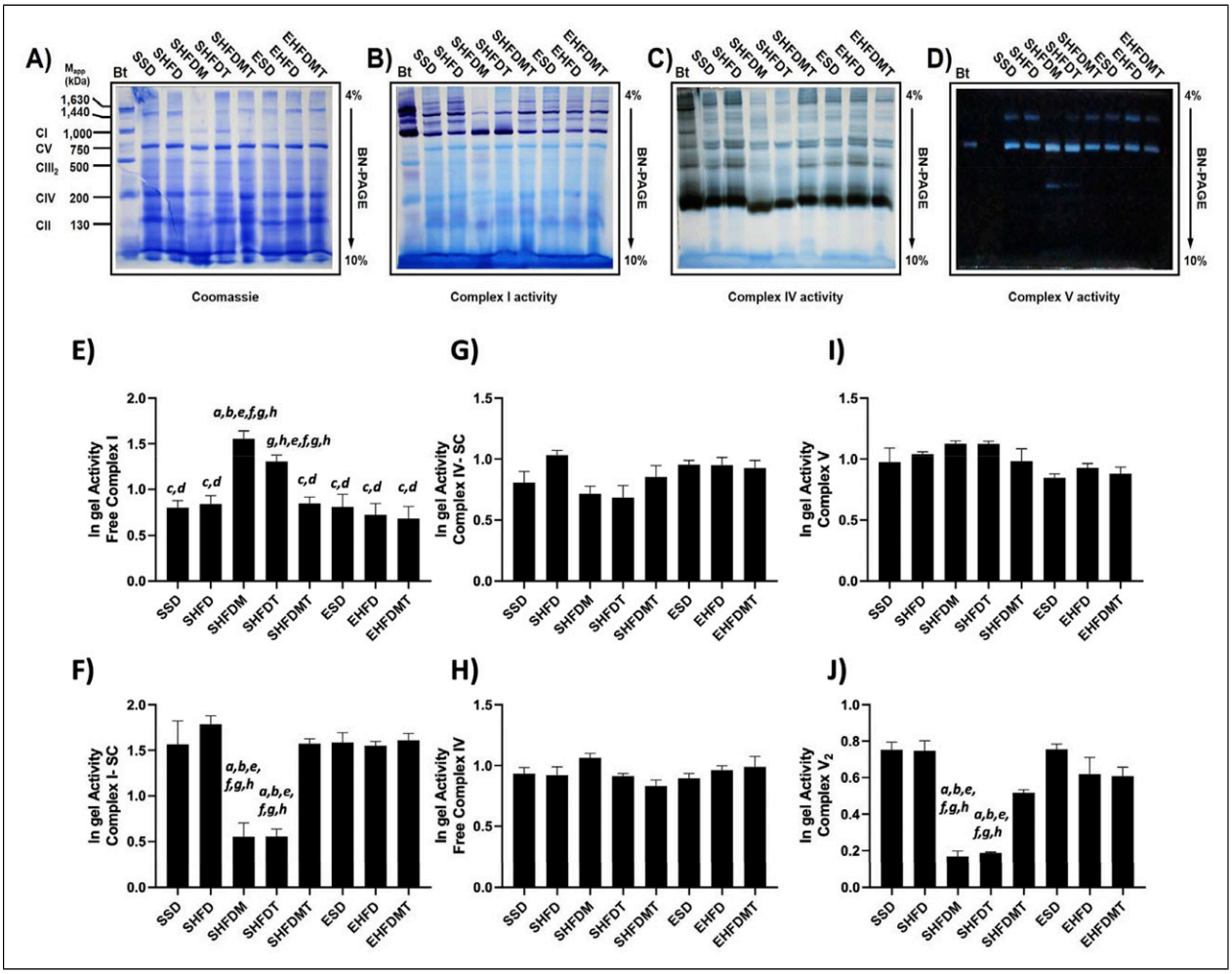

Figure 2. The in-gel activity of complexes and supercomplexes solubilized with digitonin. The respiratory complex and supercomplexes of rat liver mitochondria were solubilized with digitonin and analyzed by BN-PAGE. Protein Coomassie-stained (A), the in-gel activity of the complex I (B), complex IV (C), and complex V (D). Densitometry analysis of each complex activity was performed separately as free-complex and associated to the supercomplex. Free-complex I (E); complex I associated in supercomplexes (F); free-complex IV (G); complex IV associated in supercomplexes (H); monomer of complex V (I); and dimer of complex V (J). The intensity of activity was normalized with the Coomassie gel staining (Figure 2A). The comparisons were established using a multivariate test followed by the Holm-Sidak with a $P < 0.05$. The letters represent the significant difference of the groups, i.e. (A) SSD; (B) SHFD; (C) SHFDM; (D) SHFDT; (E) SHFDMT; (F) ESD; (g) EHFD; (h) EHFDMT.

Membrane potential ($\Delta\Psi$) is the link between mitochondrial ATP synthesis and the electron chain activity and it is the principal component for mitochondrial ATP synthesis.[54,55] Figure 4D shows the $\Delta\Psi_m$ generation using succinate as substrate. The groups that generated the highest $\Delta\Psi_m$ were those fed with an SD (SSD, black line), but the fastest rate of $\Delta\Psi$ generation was found in the group that exercised as well (ESD, magenta line); the slower rate was seen in the sedentary group fed with HFD (SHFD, Figure 4D). The rate of $\Delta\Psi_m$ generation (Figure 4E) was 30% lower in the SHFD than in the SSD group, suggesting that HFD produced a negative impact in the sedentary rats. However, the treatment with MTF (SHFDM), tBHQ (SHDFT), and both given simultaneously (SHFDMT) showed recovery of the velocity of $\Delta\Psi_m$ generation. Interestingly, the EHFD and EHFDMT groups exhibited an increase in the speed of $\Delta\Psi_m$ generation of 26% and 38% in comparison to the SSD group, and 1.7 and 1.8 times relative to the SHFD group.

Mitochondrial ATP synthesis was quantified in the same experimental conditions used in the $\Delta\Psi_m$ assay (Figure 4F). The SHFD and SHFDM groups significantly diminished the ATP synthesis. Contrariwise, the SD-fed animals that exercised (ESD) and the HFD-fed rats with the double (SHFDMT) and triple treatments (EHFDMT) improved the ATP synthesis with respect to SHFD, and reached the value of the SSD group.

ROS production is an important parameter related to mitochondrial damage. The only group that significantly decreased ROS production was the SHFDM group (25% less than SSD) (Figure 4G). Notably, isolated mitochondria from

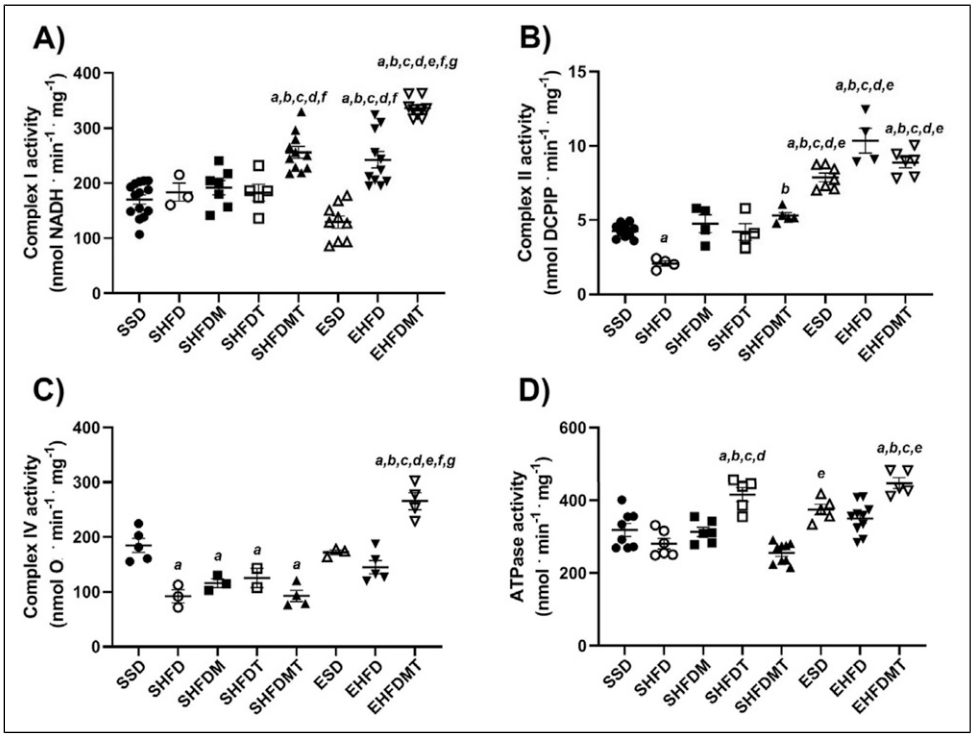

Figure 3. The activity of OXPHOS complexes from isolated rat liver mitochondria with the different treatments. The NADH oxidation by complex I (A), DCPIP reduction by complex II (B), oxygen consumption by complex IV (C), and ATP hydrolysis by complex V (D) were quantified as described in the material and methods. The comparisons were established using a multivariate test followed by the Kruskal-Wallis test with post-hoc Dunn test $P < 0.005$. The letters represent the significant difference of the groups, i.e. (A) SSD; (B) SHFD; (C) SHFDM; (D) SHFDT; (E) SHFDMT; (F) ESD; (G) EHFD; (H) EHFDMT.

the ESD group produced 20% more ROS than the SSD group ($P < 0.05$); nevertheless, this effect was reversed when the animals were fed with an HFD, regardless of supplementation with MTF and tBHQ. Since the relationship between ROS production and the electron flux in the respiratory chain is the electron leak (eL), we determined it as the ratio between the H_2O_2 production rate (Figure 4G) and the oxygen uptake rate (Figure 4A), using the following equation: Electron leak (%) = $(V_{H2O2} \cdot 100)/V_{oxygen\ uptake}$ (Figure 4H). The eL for the SSD group was 1.9% of the maximal mitochondrial oxygen uptake (Figure 4A). The HFD significantly increased the eL to 3.1% in the SHFD group, while the interventions in the sedentary animals reduced it to 2.2 % (SHFDM), 2.5% (SHDFT), and 2.7% (SHFDMT). Notably, the effect of the HFD was prevented by the exercise routine: the ESD and EHFD showed an eL value of 2.2% and 2.3%, respectively. The triple treatment (EHFDMT) had an eL value of 1.1%. The results suggest that as the respiratory activity rises, a rapid decrease in the rate of $O_2^{\bullet-}$ generation (detected as extramitochondrial H_2O_2) occurs.

Discussion

Obesity and age are known risk factors associated with multiple metabolic diseases such as diabetes, coronary artery disease, cancer, and non-alcoholic fatty liver disease. Therefore, finding therapeutic interventions to try to halt or ameliorate them, are an important and paramount current health challenge in order to improve people's quality of life, their outcomes and decrease massive health care expenditures. Previously, we published the positive effects of the triple treatment on lifespan and health span[36]; however, an outcome that was not explored in that study is the effect of the triple treatment on the liver mitochondrial physiology, even though a preservation of the hepatic cytoarchitecture was indeed observed.

Here we have demonstrated that when mitochondrial respiration was evaluated, the activity of the OXPHOS complexes decreased in the HFD sedentary group (SHFD) in agreement with other studies.[56] Mitochondrial respiratory complexes are assembled into respiratory supercomplexes or respirasomes. The formation of these higher-order structures brings together complex I, a dimer of complex III, and complex IV, facilitating electron transport and minimizing electron leakage and ROS generation.[57,58] Our results show that the amount of mitochondrial supercomplexes in the SHFDM and SHFDT groups was reduced and the free form of CI, CIV, and CV increased, concurring with previous studies that reported that fat consumption modifies the inner mitochondrial membrane altering the mitochondrial electron transport supercomplexes assembly and impairing oxidative phosphorylation.[56,59] Interestingly, these groups were the ones

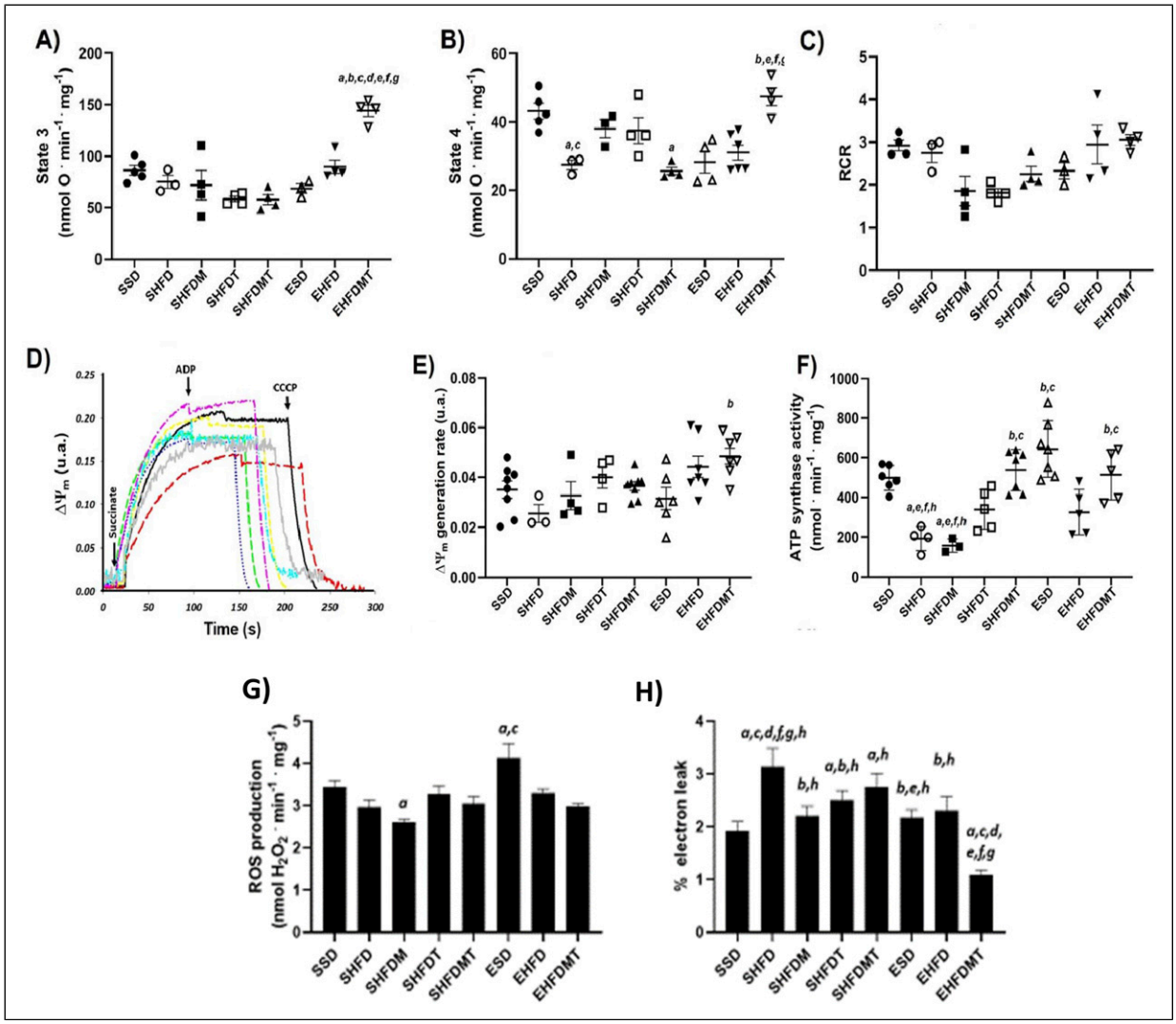

Figure 4. Evaluation of the mitochondrial bioenergetics' parameters of isolated rat liver mitochondria. Oxygen consumption in state 3 (A) and state 4 (B) of respiration was induced by the addition of ADP using succinate as oxidizing substrate. The RCR (C) was obtained from the ratio of oxygen consumption in state 3/oxygen consumption in state 4. Mitochondrial transmembrane potential ($\Delta\Psi_m$) was determined (D) using safranin O following absorbance changes at 511-533 nm. The $\Delta\Psi_m$ was collapsed by adding 5 μM CCCP (Spectrophotometric record color: SSD-black; SHFD-red; SHFDM-green; SHFDT-yellow; SHFDMT-blue; ESD-magenta; EHFD-cyan; EHFDMT-gray). Velocity rate $\Delta\Psi_m$ was determined (E). The mitochondrial ATP synthesis (F) was measured by the change in absorbance of NADPH. Production of H_2O_2 (G) was determined using succinate + potassium cyanide and Amplex® Red Hydrogen Peroxide Assay Kit. The electron leak (H) was determined using the data of H_2O_2 production and oxygen uptake in state 3. The comparisons were established using a multivariate test followed by the Kruskal-Wallis test with post-hoc Dunn test $P < 0.005$. The letters represent the significant difference of the groups, i.e. (A) SSD; (B) SHFD; (C) SHFDM; (D) SHFDT; (E) SHFDMT; (F) ESD; (G) EHFD; (H) EHFDMT. The comparisons were established using ANOVA and post hoc Dunn $P < 0.05$.

with the lower ATP synthesis and oxygen consumption in state 3 of respiration and the greater eL. This could be related to a lower content and activity of CI which have been related to an increase in mitochondrial hydrogen peroxide and superoxide production.[60-62]

Obesity is also associated with liver mitochondrial dysfunction and clearance, leading to lower mitochondrial content and impaired bioenergetic function. There are reports that SIRT3 is a crucial regulator of mitochondrial function, which controls global acetylation of the organelle. Mice fed with HFD have shown low SIRT3 activity, as well as impaired mitochondrial function, especially the oxygen consumption rate and CI activity, along with protein hyperacetylation in the liver.[63] In another study, mice lacking SIRTt3 and fed with HFD developed accelerated obesity, insulin resistance, and steatohepatitis compared to wild-type mice.[64] Together, these data suggest that CI dysfunction could be due to increased acetylation of this protein caused by decreased SIRT3 expression. However, in those

animals that exercised, the opposite effect has been reported,[65,66] suggesting that the animal that received a triple treatment improved their CI activity, as well as their ATP synthesis, and mitochondrial membrane potential.

Goncalves and collaborators found in a mice model that exercise improved mitochondrial structure, respiration and increased the expression of cytochrome C in abnormal liver mitochondria.[67,68] Other studies also reported changes in the activity of liver mitochondrial complexes in response to exercise, particularly complexes I, IV and V, followed by an increase in mitochondrial glutathione content and no increase in ROS production.[69,70]

It has been recently reported that liver mitochondria preferentially oxidizes succinate[71,72] and that there is a dysfunction in complex II activity in mitochondria isolated from HFD-rat livers.[73] Complex II is considered an important factor in mitochondrial respiration regulation and a decrease in its activity has been associated with a reduction in mitochondrial respiration and an increase in ROS production.[73] Using succinate as a substrate, we also found a decrease in mitochondrial oxygen consumption, membrane potential rate generation, and ATP synthesis in HFD-rats.

It is known that exercise is an inducer of the hormetic response and that the adaptive response that occurs is systemic.[74] Hormesis has been defined as a process in which intermittent exposure to low doses of a physical, chemical, or environmental harmful stimulus, can induce a beneficial response to counteract or adapt to further toxic stimuli.[31,75,76] Among the main signaling mechanisms that occur during exercise-induced hormesis is the regulation of ROS, which can vary in type and abundance, and these, in turn, depend on the type of exercise routine and time of performance.[77-79] Our results showed that the ESD group was the only one that increased H_2O_2 levels while the SHFDM group lowered them. Interestingly, the electron leakage in the sedentary groups increased compared to the SSD control, except for the SHFD group. The EHFDMT group showed the lowest electron leakage compared to all the experimental groups. The other exercise groups presented similar values to the SSD group, nevertheless, they showed differences concerning the SHFD group. This can be explained under the concept of hormesis. It is clear that in the ESD group, the increase in H_2O_2 concentration is related to the protection and repair of the oxidative damage produced by the exercise routine itself. Different research groups have reported that performing high, medium, and low-intensity exercise routines at different times, increases the antioxidant response by a mechanism involving the Nrf2/ARE signaling pathway.[80,81] Moreover, Yavari et al,[82] 2015 demonstrated that mitochondrial ROS produced during regular exercise is necessary to activate primary signaling pathways associated with muscle adaptation. However, although we found that there is an increase in PGC1α and AMPK, along with a better respiratory capacity, the survival of this group after 24 months was only 50%. Food restriction has been shown to increase longevity in several species, including rats,[83,84] but intensive exercise along with caloric restriction in rats is known to increase mortality.[85] Even though our animals were not under caloric restriction, it is possible that the SD was not providing sufficient nutritional input to carry out the physical activity or that rats did not increase their food intake to compensate for the increased energy expenditure, since the EHFD group increased his life expectancy by 8% compared to ESD. The triple-treatment (EHFDM) reversed the damage generated by HFD by increasing the amount and activity of OXPHOS complexes, increasing the rate of membrane potential generation, as well as ATP synthesis, thus achieving 77% survival.

The triple treatment increased the expression of AMPK and PGC1α without a concomitant increase in H_2O_2 production but was associated with a decrease in electron leakage, suggesting that the triple treatment induced a synergistic effect of exercise with MTF and tBHQ on the hormetic response, thus improving oxidative stress response, reducing mitochondrial dysfunction, and exhibiting hepatoprotective effects against the toxicity generated by HFD. It has been reported that MTF can improve mitochondrial respiratory activity by a mechanism involving AMPK activation in the liver of HFD-fed mice. Its euglycemic effect has been related to the inhibition of hepatic glucose production. The main mechanism of MTF is the activation of AMPK due to decreased intracellular ATP content; probably through inhibition of complex I.[26,86,87] It is important to emphasize that the beneficial effects of MTF are actually dose-dependent.[72] MTF inhibits complex I only if concentrations are higher than 1 mM.[88] This concentration was not reached in the liver in our model. At therapeutic doses used in diabetic patients, the concentration of MTF in the liver fluctuates between 40 and 70 μM.[89,90]

Our results did show a decrease in ATP synthesis in the SHFDM group (Figure 4), however, we did not find inhibition of complex I (Figure 3), neither did we see a reduction in OXHPOS complex activity or mitochondrial membrane potential in the MTF-treated groups, but we were able to determine an increase in AMPK levels which could be due to a mechanism independent of mitochondrial CI inhibition. Another postulated explanation might be the use of fat as a fuel for thermogenesis. Previously in this same model, a decrease in body weight and fat of treated rats was observed,[35] so the decrease in ATP levels and the increase in the ADP/ATP ratio which results in the activation of AMPK may be related to the utilization of lipids to generate thermogenesis. Another mechanism might be related to changes in mitochondrial dynamics since it has been reported that MTF can induce mitochondrial fission through AMPK-Mff signaling, improving mitochondrial respiration and decreasing hyperglycemia in obesity.[72] Likewise, allosteric inhibition of fructose 1,6-bisphosphatase and mitochondrial glycerol-3-phosphate dehydrogenase by MTF could be another mechanism involved in AMPK activation.[91] In that case, MTF could also enhance hepatic metabolism through activation of AMPK and PGC1α (Figure 1), which in turn activates various signaling pathways such as mitophagy and mitochondrial biogenesis.

tBHQ is one of the most recognized inducers of the Nrf2-ARE signaling pathway.[51] Jiang et al,[92] 2022 reported that tBHQ treatment decreases renal damage by decreasing mitochondrial ROS production and improving mitochondrial function in HFD-induced obese rats since the Nrf2/ARE pathway is triggered by modulating mitochondrial and cytoplasmic redox state. So, one possible explanation is that tBHQ may prevent the $\Delta\Psi_m$ collapse by ROS scavenging and by activating Nrf2, increasing antioxidant enzyme expression and enhancing mitochondrial metabolism. Furthermore, Nrf2 has also been reported to regulate the expression of genes that promote mitochondrial biogenesis, such as mitochondrial transcription factor A (TFAM), and thus is directly involved in mitochondrial preservation. AMPK activation can reduce NAFLD by suppressing *de novo* lipogenesis in the liver, increasing fatty acid oxidation, and preventing mitochondrial damage through the regulation of mitochondrial fusion, autophagy, cell metabolism, and mitochondrial biogenesis. In addition to Nrf2 activation through tBHQ, exercise, and MTF might add their protective effect by activating AMPK. Activation of hepatic AMPK leads to inhibition of acetyl CoA carboxylase and consequently to increased fatty acid oxidation. AMPK also promotes mitochondrial remodeling in response to physical exercise and MTF, and may stimulate the expression of PGC1α, a master regulator in mitochondrial biogenesis.[93-96] Moreover, the HFD-rats treated with the two chemicals without exercise (SHFDMT) also showed the beneficial effect of an increase in the expression and activity of OXPHOS complexes and recovery of ATP synthesis. This might be explained due to the synergistic effect in which MTF activates AMPK and mitochondrial turnover, while tBHQ activates the Nrf2 pathway.[29] Moreover, it has been reported that myristoylation of the β subunit in AMPK stimulates its mitochondrial localization, where it could regulate removal of damaged mitochondria by mitophagy.[96]

All of the above findings must be considered in order to understand the possible interactions and/or synergistic effects among exercise, MTF, and tBHQ role in regulating mitochondrial function. Some contradictory reports combining treatments of MTF and exercise, abrogated the benefits of exercise on mitochondrial function.[97,98] On the other hand it has been also reported that insulin sensitizing by exercise and metformin treatment restores brain mitochondrial function in insulin-resistant states[99] and that the combined treatment of MTF and exercise tightly coupled mitochondrial function in rats quadriceps muscle,[100] suggesting a synergistic effect. In a previous study, our group found that the combined treatment of MTF and exercise improved the overall rats physiology and endurance, but did not recover the redox homeostasis, especially during old age.[101] Therefore, to grasp the synergy of the triple treatment it is imperative to evaluate all involved variables during middle age and aging, when the antioxidant systems decrease in quantity and quality. So that the ROS generated by the hormetic effect of exercise and MTF may not be buffered and the eustress redox state could become distress[102] generating cellular damage. Thus, tBHQ treatment might enhance that component of the response.

Another important aspect to be considered in order understand the synergy of the triple treatment is the hormetic effect. In fact, the drug treatments were not continuous and no classical aerobic exercise was performed. In the previous studies cited above, it was found that treatment with MTF from 18 to 24 months (the last 6 months of rats life) generated a better effect than the continuous treatment from 12 to 24 months (1 year treatment), so that the way the drugs were administered in this study (only 1 week per month) may be activating cellular and mitochondrial response in a hormetic manner without allowing the system to adapt. The same can be said for exercise, the Fartlek-type routine varies in intensity in a way that challenges the system more than a classic exercise routine, strengthening the hormetic response.

To conclude, and to integrate, comprehend, and compare the effects of exercise together with the MTF and tBHQ treatments, we analyzed three important parameters: electron leakage (eL), survival, and ATP synthesis (Figure 5). The SSD group (the black circle in Figure 5) represents control determinations for eL, survival, and ATP synthesis. The worst-case scenario was for the animals that were fed with an HFD and received no interventions (SHFD, white circle in Figure 5), where only 25% of the rats survived, and showed a high eL, and a decreased ATP synthesis. A better scenario was represented by the exercised groups fed with SD (ESD group, blue square in Figure 5), which showed the highest values on ATP synthesis. The administration of HFD with an exercise routine (EHFD, fuchsia diamond in Figure 5) increased the eL and their life expectancy, but ATP synthesis decreased by half with respect to the ESD group; Nevertheless, this group performed better than the SHFD group, as there was still 1.5-fold higher ATP synthesis relative to the non-exercising SHFD group. Intriguingly, treatment with tBHQ alone (SHFDT group, green triangle in Figure 5) behaved like the EHFD group, in contrast to the treatment with MTF alone (SHFDM group, red triangle in Figure 5), which only increased survival by 66% and increased eL by 21% compared to the SSD group. The combination of MTF + tBHQ in HFD rats (SHFDMT, yellow square in Figure 5) increased ATP synthesis 2.9-fold and survival by 66%. ATP synthesis was 1.2-fold larger than in the SD group (SSD, black circle, Figure 5) and was almost as effective as the triple therapy. Triple treatment (EHFDMT, light blue diamond in Figure 5), was the best intervention for HFD rats, increasing ATP synthesis 1.7 times and 77% survival and decreasing the by 40% eL. It is important to state that almost any mitochondrial stress activates AMPK, including OXPHOS complexes inhibition or molecules that decrease ATP synthesis (such as MTF); then, AMPK acts as a mitochondrial homeostasis sensor and an activator of cell metabolism.[103,104]

It must be acknowledged that there are several limitations of the current study. Although AMPK-PGC1α was evaluated, we still needed to evaluate the complete PPARγ-PGC1α-

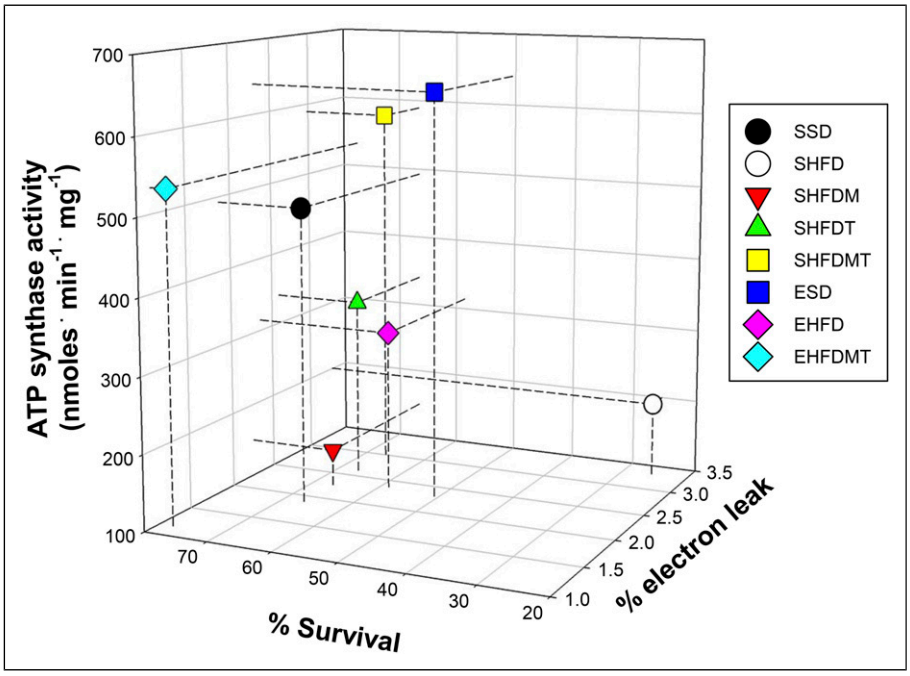

Figure 5. Exercise and triple therapy reverse the effect of a High-Fat-Diet in rat liver mitochondria. (Black circles) SSD; (white circles) SHFD; (red triangles) SHFDM; (green triangles) SHFDT; (yellow squares)SHFDMT; (blue squares) ESD; (fuchsia diamonds) EHFD; (light blue diamonds) EHFDMT.

Sirt3 axis. Another limitation we found is that we were unable to obtain samples to quantify mitochondrial morphology using electron microscopy. However, we identified the relationship between exercise and triple treatment in ATP synthesis, electron leak and survival.

Conclusion

In summary, exercise is an excellent intervention to prevent liver damage caused by HFD, but when combined with intermittent tBHQ and MTF treatment, there is a greater improvement to counteract the metabolic, oxidative, and inflammatory effects that occur in HFD-induced obesity. Since we found an interesting hormetic effect on the redox state and oxidative damage to biomolecules, it would be an intriguing proposition for future studies to further analyze this effect.

Finally, it should not be forgotten that humans have a unique physiology and, especially, social behaviors, very different from rodents. Evidently more studies are needed to be able to extrapolate this type of interventions to humans. Still, the results are highly encouraging.

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Author Contributions

Stefanie Paola López-Cervantes, Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. Rafael Toledo-Pérez, Methodology, Investigation. Jaime Abraham De Lira-Sánchez, Methodology, Investigation. Giovanni García-Cruz, Methodology, Investigation. Mercedes Esparza-Perusquía, Methodology, Investigation. Armando Luna-López, Conceptualization, Methodology, Investigation, Review & editing. Juan Pablo Pardo, Data curation, Validation, Review & editing. Oscar Flores-Herrera, Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision, Funding acquisition. Mina Konigsberg, Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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