Quercetin Protects Rat L6 Myocytes from Antimycin A-Induced Mitochondrial Dysfunction

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Abstract

Objective: Mitochondrial dysfunction is often associated with various disorders such as diabetes, Alzheimer's etc. Reactive oxygen species (ROS), aging, and reduction of mitochondrial biogenesis contribute to mitochondrial dysfunction. Antimycin-A (AMA) damages the mitochondria through inhibition of mitochondrial electron transport. The present study sought to investigate effects of quercetin on rat L6 cells and whether quercetin protects mitochondria against oxidative damage caused by AMA.

Methods: Rat L6 myocytes were used for the study. Effects of quercetin on Antimycin-A induced mitochondrial dysfunction was studied using cytotoxicity, ATP levels, mitochondrial superoxide production and NDUFB8 mRNA expression.

Results: In this study, exposure of L6 myocytes to AMA induced an increase cell death, decreased ATP content, followed by a decrease in mitochondrial superoxide, and decreased expression of NDUFB8. We found that quercetin protected myocytes from antimycin-A (AMA) induced L6 cell death as evidenced from increased lactate dehydrogenase (LDH) leakage into extracellular medium, protected ATP production, prevented increase in oxidative stress and restored levels of NDUFB8 mRNA expression implying improved mitochondrial function.

Conclusion: These results suggest that the quercetin showed protective effect against AMA-induced mitochondrial dysfunction by increasing ATP production, decreasing oxidative stress, and restoring mitochondrial function.

Keywords: Quercetin; Mitochondrial biogenesis; Oxidative stress; Antimycin-A; Cytotoxicity; ATP; Myocytes

Introduction

Mitochondrial biogenesis is an important process responsible for regulating energy demand of the cell. Accordingly, it is affected by a variety of factors, like exercise, low temperature, and oxidative stress [1,2]. Mitochondrial biogenesis is a type of mitochondrial dysfunction and can play a role in many diseases, like diabetes and Alzheimer's, etc. [3,4]. Increased oxidative damage and inflammation can cause mitochondrial damage that may lead to serious acute and chronic pathologies such as multi organ failure, neurodegeneration, cardiovascular diseases, and cause aging. Mitochondrial biogenesis enhance cellular function and survival *in-vitro* and *in-vivo* and promote cellular recovery from damage caused by adverse environmental, pathophysiological, and/or infectious agents [4,5]. Mitochondrial biogenesis is a complex process that includes growth and division of pre-existing mitochondria, ultimately leading to increased energy production in cells. High energy status most likely determines the outcome of cellular insult caused by oxidative stress, chemical or mechanical injury, inflammation, and routine tissue repair [6].

Due to the large number of mitochondria myocytes play an important role in glucose uptake and burning it into ATP. Beneficial effects of caloric restriction, diet, and physical exercise have been noted in diabetes, some of these effects are attributed to mitochondrial biogenesis as newly formed mitochondria are more efficient in terms of energy utilization [7]. Number of chemicals from plant origin and plant extracts has been shown to increase mitochondrial biogenesis [3]. Polyphenols represents beneficial group of naturally occurring compounds with hypoglycemic potentials. These are the most abundant antioxidants in the human diet and are commonly found in onions, apples, tea, coffee, red wine, etc. [8]. Chemically, flavonoids can be widely classified into different categories such as flavanols, flavones, catechins, flavanones, etc. [9]. Quercetin is the most well researched of all flavonoids and it is one of the most potent scavengers of reactive oxygen species including superoxide and reactive nitrogen species [10]. Quercetin has also been reported to exhibit anti-oxidative, antitumor, anti-inflammatory, vasodilatory effects [11]. Besides ROS scavenging activity it has been shown to promote mitochondrial biogenesis and improving insulin sensitization in various *in-vivo* and *in-vitro* studies [12]. Quercetin has been shown to promote mitochondrial biogenesis in brain and muscle and improve exercise tolerance [13,14].

In mitochondrial respiratory chain, Complex I and III are major sources of intracellular superoxide and oxidative stress [15]. Antimycin-A is an inhibitor of complex III. The binding of AMA to complex III is directly associated with inhibition of its enzymatic activity [16,17] thus increasing ROS formation through complex I and

II [18]. Mitochondrial biogenesis has been studied in muscle cell lines as well as in animal models, however these cells has not been used as model to screen chemicals for mitochondrial biogenesis. Thus in the present study, we intended to investigate protective effects of quercetin on mitochondria, ATP synthesis and complex I mRNA expression with a view to develop a model to study mitochondrial biogenesis and to identify chemicals of plant origin that cause mitochondrial biogenesis. We demonstrate that quercetin shows protective effects on oxidative stress induced myocyte cell death through increasing ATP synthesis- an indirect but ultimate indicator of mitochondrial biogenesis. An understanding of the mechanisms underlying mitochondrial biogenesis may facilitate the development of therapeutics in diseases involving mitochondrial dysfunction.

Materials and Method

Materials

Quercetin, Antimycin-A and DMSO were obtained from Sigma Chemical Co (St Louis, MO). PenStrep and DMEM medium were obtained from VWR international (Randor, PA), FBS was obtained from ATCC (Manassas, VA), MitoSOXTM Red Mitochondrial super oxide indicator was obtained from Thermofisher (Grand Island, NY). Trypsin-EDTA was purchased from Invitrogen (Carlsbad, CA). CytoTox-ONE Homogenous Membrane Integrity Assay kit and CellTiter-Glo luminescent cell viability assay kit (ATP) were obtained from Promega (Madison, WI). The BCA protein assay kit was purchased from Pierce (Rockford, IL). All other reagents were of analytical grade and were purchased from commercially available vendors.

Cell culture

Rat L6 were obtained from ATCC and expanded in laboratory. Cells were grown in DMEM media with glutamate, supplemented with 10% FBS and antibiotics (PenStrep). For fluorescence and for luminescence assays that involved a microplate reader, the cells were seeded in flat micro-clear bottom 96-well plates (BD Falcon, BD Biosciences, San Jose, CA) at a density of 3×10^5/mL. All drugs were either directly added to the culture medium or dissolved in dimethyl sulfoxide (DMSO) and then added to the culture medium (final DMSO concentration was 0.1% v/v). Plates were incubated at 5% CO_2, 37°C and 95% relative humidity for 24 or 48 h time period.

Determination of cell injury

Release of lactate dehydrogenase (LDH) into the extracellular medium was used as an indicator of cytotoxicity. The relative activity of LDH in the medium (as percentage of total intracellular plus extracellular activity) was determined using a CytoTox-ONE Homogenous Membrane Integrity Assay kit. In the presence of LDH, resazurin is converted to resorufin which was quantified in a microplate reader (excitation 560 nm, emission 590 nm). Enzyme activity in the medium was sampled at indicated time points and expressed as percentage of total intracellular and extracellular LDH activity.

Determination of cellular ATP content

For determination of ATP content we used a CellTiter-Glo Luminescent cell viability kit. Luciferin is mono-oxygenated in the presence of ATP, Mg^{2+}, and molecular oxygen to luminescent compound oxyluciferin. Briefly, after treatment and incubation with test chemicals, cells were treated with premixed CellTiter-Glo reagent, then plates were incubated for 10 min at room temperature to stabilize luminescent signal. Luminescence emitted out by oxyluciferin is then measured with luminometer.

Determination of cell number

Measurement of monolayer protein content and actual cell count over time were used to estimate cell number. Cells were seeded in 6-well plates at a concentration of 1.2×10^6 cells/well. Cells were treated with either 0.1% v/v DMSO or 50 μM quercetin and incubated for 24 h time period at 5% CO_2, 37°C and 95% relative humidity. To determine protein concertation L6 myocyte monolayers were washed with PBS solubilized in Triton buffer (0.05% Triton X-100, 100 mM Tris-base, and 150 mM NaCl, pH 7.5). Protein concentrations, were determined by the bicinchoninic acid (BCA) method according to the manufacturer's instructions. Actual cell count was made by harvesting cells and counting using phase contrast microscope and Neubauer chamber.

Measurement of mitochondrial superoxide

Mitochondrial superoxide was measured with the cell-permeable fluorogenic probe, hydroethidine coupled to triphenylphosphonium (mito-HE, MitoSox Red®). Being a lipophilic cation, MitoSOX Red is selectively targeted to mitochondria where it specifically reacts with superoxide anion. Quercetin, AMA or quercetin and AMA preexposed cells were loaded with MitoSOX Red (1 μM) for 10 min at 37°C, washed with PBS, and then fluorescence was determined using the excitation at 396 nm and emission at 580 nm as described in Kashimshetty et al. [19]. Relative Fluorescence Units (RFUs) of untreated cells was considered as 100 percent.

Quantitative real-time PCR

NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 8 (NDUFB8) is also known as complex I, is located in the mitochondrial inner membrane. It is encoded by nuclear DNA and catalyzes the oxidation of NADH and electron transfer. Relative increase in levels of expression of NDUFB8 correlates with increased mitochondrial function [20-22]. For NDUFB8 mRNA expression, total RNA was isolated from L6 cells with RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from 1 μg of RNA template using a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Grand island, NY). PCRs were carried out by using 3 μl of 1:3 diluted cDNA template combined with Maxima® SYBR Green qPCR Master Mix (2X) at a final concentration of 1X (Thermo Fisher Scientific, Grand Island, NY.), ROX (Thermo Fischer Scientific, Grand Island, NY.) and primers (Thermo Fisher Scientific, Grand Island, NY) at a concentration of 10 nM and 400 nM respectively. For real-time PCRs primer sequences for NDUFB8 were: FW: 5'-GGC GAT CCC AAC AAA GAA CC-3'; REV: 5'-TTT CTA GGA TTG AAG GAG TC-3' and the abundance of mRNAs was normalized against tubulin FW: 5'-CTC TCT GTC GAT TAC GGC AAG-3'; REV: 5'-TGG TGA GGA TGG AGT TGT AGG-3' using the $\Delta\Delta Ct$ method. Relative change in mRNA expression was then expressed as fold change over untreated cells.

Statistical analysis

All data were expressed as mean ± SEM of at least three independent experiments. Each experiment is performed in triplicates

(N=3). Statistical analysis was performed using SPSS 20.0 (SPSS, Chicago, IL) by using one-way ANOVA followed by Tukey's post hoc test. Data were considered statistically significant at $P \leq 0.05$.

Results

Effects of quercetin on antimycin-A (AMA) induced toxicity

Cell viabilities were determined using L6 cells and LDH assay for various concentrations of quercetin. Dose dependent cytotoxicity was observed with quercetin (0-500 μM) for 24 h and 48 h time points. Significant difference in % LDH leakage has been noted at quercetin concentrations 100, 250 and 500 μM) at 24 h time point and for 50, 100, 250 and 500 μM concentrations at 48 h time point (Figure 1A).
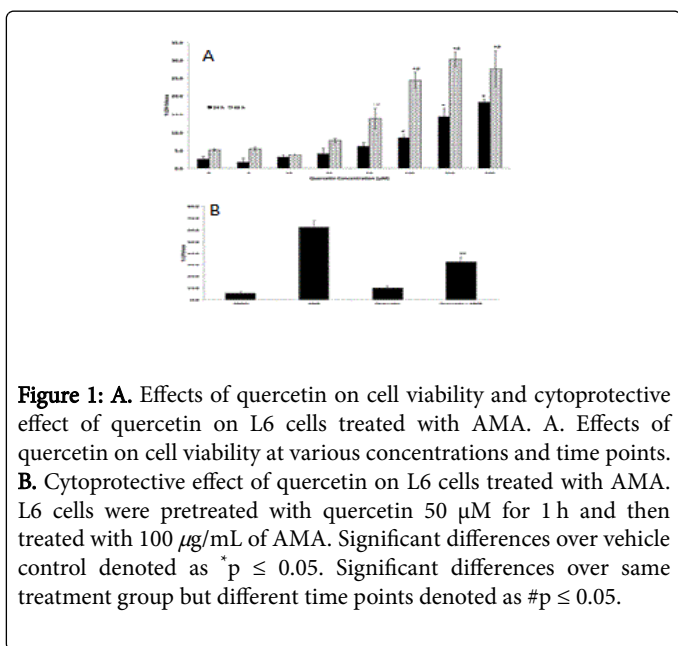

Figure 1: A. Effects of quercetin on cell viability and cytoprotective effect of quercetin on L6 cells treated with AMA. A. Effects of quercetin on cell viability at various concentrations and time points. **B.** Cytoprotective effect of quercetin on L6 cells treated with AMA. L6 cells were pretreated with quercetin 50 μM for 1 h and then treated with 100 μg/mL of AMA. Significant differences over vehicle control denoted as $^*p \leq 0.05$. Significant differences over same treatment group but different time points denoted as #p ≤ 0.05.

We used Antimycin-A to induce cell death in myocyte through mitochondrial dysfunction. We then determined the dose and time of exposure to 100 μg/mL AMA required to reduce cell viability by 50% after 24 h incubation (data not shown). Cells were pretreated with quercetin 50 μM concertation that showed no significant effects on myocyte cell death at 24 h time period. One hour (1 h) after quercetin treatment, cells were then treated with 100 μg/mL AMA in the presence of quercetin. Significant reduction in cytotoxicity (~30%) has been noted in group treated with quercetin (50 μM)+AMA compared with AMA alone (Figure 1B).

Effects of quercetin on ATP levels

To determine whether the quercetin affected energy production, we measured ATP levels. Cellular ATP levels after treatment with test chemicals were determined on L6 cells by CellTiter-Glo Luminescent cell viability kit. Significant difference in % ATP has been noted at quercetin concentrations 500 μM at 24 h and 250 and 500 μM at 48 h time point (Figure 2A). Non-significant increase in ATP levels has been noted with concentration 5, 10 and 25 μM at 48 h time point as compared to 24 h time point. To determine protective effects of quercetin on AMA-induced ATP loss, cells were then pretreated with quercetin 50 μM and then 1h after quercetin treatment cells were treated with 100 μg/mL AMA in the presence of quercetin. Quercetin

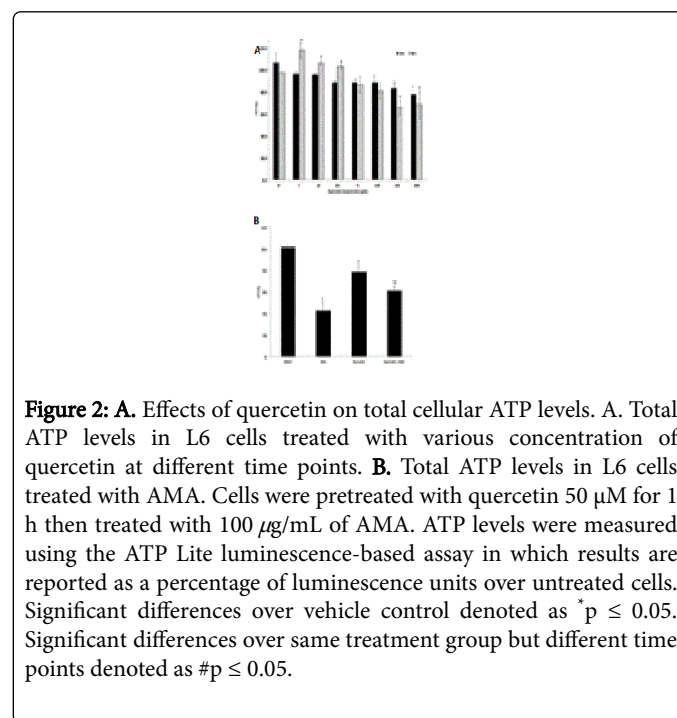(50 μM) increased cellular ATP by 18% compared to AMA alone (Figure 2B).

Figure 2: A. Effects of quercetin on total cellular ATP levels. A. Total ATP levels in L6 cells treated with various concentration of quercetin at different time points. **B.** Total ATP levels in L6 cells treated with AMA. Cells were pretreated with quercetin 50 μM for 1 h then treated with 100 μg/mL of AMA. ATP levels were measured using the ATP Lite luminescence-based assay in which results are reported as a percentage of luminescence units over untreated cells. Significant differences over vehicle control denoted as $^*p \leq 0.05$. Significant differences over same treatment group but different time points denoted as #p ≤ 0.05.

Effects of quercetin on myocyte cell number

Mitochondrial biogenesis has been identified as a compensatory response to cellular injury and often increased mitochondrial biogenesis attributed to increased cell proliferation. To determine whether cell number changed during the 24 h exposure to quercetin, the amount of cellular protein per well, a marker of cell number was measured in the presence and absence of quercetin (50 μM) after 24 h treatment. Monolayer protein content as well as cell number did not change in the presence of quercetin (Figures 3A and 3B).
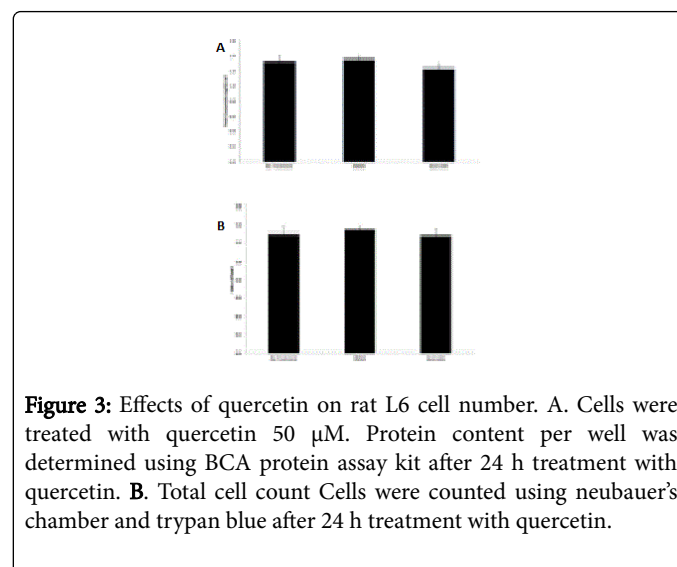

Figure 3: Effects of quercetin on rat L6 cell number. A. Cells were treated with quercetin 50 μM. Protein content per well was determined using BCA protein assay kit after 24 h treatment with quercetin. **B.** Total cell count Cells were counted using neubauer's chamber and trypan blue after 24 h treatment with quercetin.

Changes in mitochondrial superoxide levels

Quantitative measurements of mean intensity from the AMA-induced cells demonstrated a 187.8% increase compared with u cells (Figure 4).
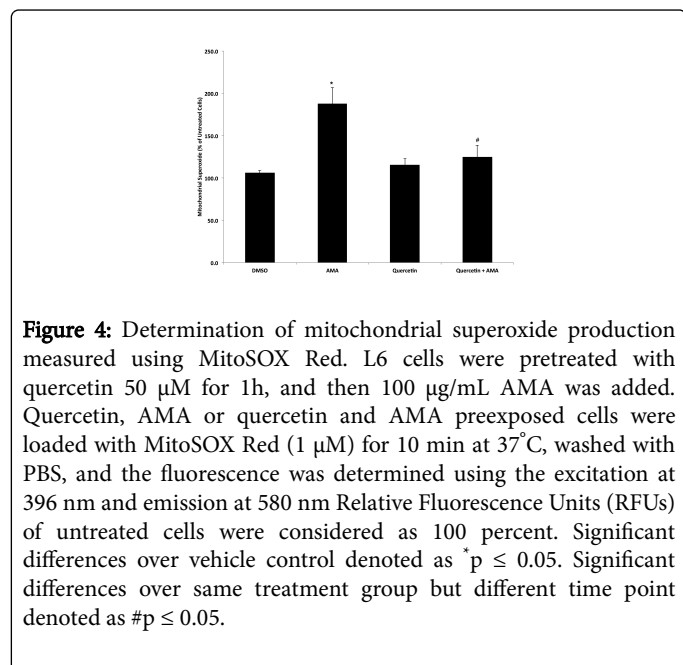

Figure 4: Determination of mitochondrial superoxide production measured using MitoSOX Red. L6 cells were pretreated with quercetin 50 μM for 1h, and then 100 μg/mL AMA was added. Quercetin, AMA or quercetin and AMA preexposed cells were loaded with MitoSOX Red (1 μM) for 10 min at 37°C, washed with PBS, and the fluorescence was determined using the excitation at 396 nm and emission at 580 nm Relative Fluorescence Units (RFUs) of untreated cells were considered as 100 percent. Significant differences over vehicle control denoted as $^*p \leq 0.05$. Significant differences over same treatment group but different time point denoted as #$p \leq 0.05$.

In contrast, mitochondrial superoxide levels were 115.5% when cells were treated with quercetin indicating lower mitochondrial superoxide levels. In the presence of quercetin+AMA, mitochondrial super oxide levels were 124.5% suggesting protective effect of quercetin on mitochondrial superoxide production.

NDUFB8 mRNA expression

We also examined electron transport chain (ETC) integrity by examining mRNA levels of NDUFB8 which is a nuclear-encoded ETC protein. We found significant increase in NDUFB8 mRNA expression in Quercetin+AMA treated cells over cells treated with AMA alone (Figure 5).

Discussion

Mitochondrial dysfunction plays an important role in cellular injury, recovery after tissue damage, and pathogenesis of various disorders and conditions such as Alzheimer's, aging, development of insulin resistance in diabetes etc. Preventing mitochondrial dysfunction by increasing mitochondrial biogenesis is important in reducing cellular damage. In the present study, we focused on the flavonoid quercetin for its effect on prevention of mitochondrial dysfunction. We used AMA to induce mitochondrial dysfunction and to induce cytotoxicity. In our study, cell viability was increased by the addition of quercetin in AMA treated cells over just AMA treated cells. Hese results agree with the findings of others that quercetin protects against toxicant-induced cell damage [14,23-26]. Mitochondrial dysfunction induced by AMA results in decreased ATP production. Oxidative stress increases respiration and generation of ROS, resulting in ATP depletion [27]. AMA causes oxidative stress and mitochondrial dysfunction by binding to the matrix side of complex III and inhibiting the mitochondrial electron transport system [28]. We show here that

treatment with quercetin in AMA exposed cells significantl prevented loss of ATP, suggesting that quercetin protect cells from mitochondrial dysfunction
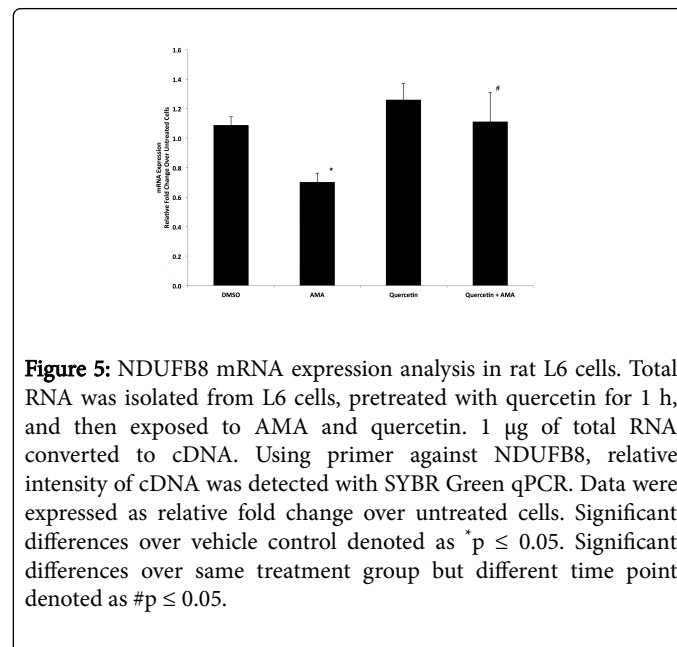

Figure 5: NDUFB8 mRNA expression analysis in rat L6 cells. Total RNA was isolated from L6 cells, pretreated with quercetin for 1 h, and then exposed to AMA and quercetin. 1 μg of total RNA converted to cDNA. Using primer against NDUFB8, relative intensity of cDNA was detected with SYBR Green qPCR. Data were expressed as relative fold change over untreated cells. Significant differences over vehicle control denoted as $^*p \leq 0.05$. Significant differences over same treatment group but different time point denoted as #$p \leq 0.05$.

Mitochondrial ROS production is intimately linked to mitochondrial dysfunction, aging, insulin resistance, exercise tolerances, etc. [29]. Our data presented here show that cells treated with quercetin exhibited decreased mitochondrial super oxide production. The MitoSOX Red results indicate that mitochondrial superoxide was increased in AMA-induced cells. Cells treated with quercetin prevented AMA-induced superoxide production by mitochondria. NDUFB8 is a nuclear encoded protein of electron transport chain. In the present study we demonstrated that AMA significantly reduced NDUFB8 expression. On the other hand cell pretreated with quercetin and then treated with AMA showed increased NDUFB8 expression without causing appreciable increase in cell number implying improved mitochondrial function as evidenced from increased ATP content. Further newly synthesized or divided mitochondria works efficiently as quercetin+AMA treated cells showed comparatively lower super oxide production over cells treated with AMA alone. Mitochondrial dysfunction, characterized by a decline in cellular ATP, cytotoxicity, and superoxide generation is central to the execution of myocyte cell death and thus increasing glucose metabolism in muscle cells to effectively burning glucose to ATP in mitochondria without generating overtly more superoxide is important. Therapeutic strategies to increase mitochondrial biogenesis and reduce oxidative stress would therefore be useful in diseases that are associated with mitochondrial dysfunction.

Conclusion

In conclusion, quercetin protects AMA-induced cell death. These results suggest that the protection of cell death is a result of increased mitochondrial biogenesis as evidenced from improved mitochondrial function through reduced oxidative stress, increased ATP levels and increased nuclear encoded NDUFB8 mRNA expression. Rat L6 myocytes in conjunction with direct markers of mitochondrial biogenesis, and with further understanding of mitochondrial biogenesis will be an useful tool to screen and identify new chemicals,

particularly of plant origin that cause mitochondrial biogenesis. The identification of novel molecules based on mitochondrial biogenesis principle may play an important role in the treatment and prevention of diseases associated with mitochondrial dysfunction.

Acknowledgement

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