

# Astaxanthin but not quercetin preserves mitochondrial integrity and function, ameliorates oxidative stress, and reduces heat-induced skeletal muscle injury

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## Abstract

Heat stress causes mitochondrial dysfunction and increases mitochondrial production of reactive oxygen species (ROS), both of which contribute to heat-induced skeletal muscle injury. In this study, we tested whether either astaxanthin or quercetin, two dietary antioxidants, could ameliorate heat-induced skeletal muscle oxidative injury. In mouse C2C12 myoblasts exposed to 43°C heat stress, astaxanthin inhibited heat-induced ROS production in a concentration-dependent manner (1–20 μM), whereas the ROS levels remained high in cells treated with quercetin over a range of concentrations (2–100 μM). Because mitochondria are both the main source and a primary target of heat-induced ROS, we then tested the effects of astaxanthin and quercetin on mitochondrial integrity and function, under both normal temperature (37°C) and heat stress conditions. Quercetin treatment at 37°C induced mitochondrial fragmentation and decreased membrane potential ( $\Delta\Psi_m$ ), accompanied by reduced protein expression of the master regulator of mitochondrial biogenesis peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ). It also induced cleavage of mitochondrial inner-membrane fusion protein OPA1. In contrast, astaxanthin at 37°C increased protein expression of PGC-1 $\alpha$  and mitochondrial transcription factor A (TFAM), and maintained tubular structure and normal  $\Delta\Psi_m$ . Under 43°C heat stress conditions, whereas quercetin failed to rescue C2C12 cells from injury, astaxanthin treatment prevented heat-induced mitochondrial fragmentation and depolarization, and apoptotic cell death. We also isolated rat flexor digitorum brevis myofibers and confirmed the data from C2C12 myoblasts that astaxanthin but not quercetin preserves mitochondrial integrity and function and ameliorates heat-induced skeletal muscle injury. These results confirm that mitochondria may be a potential therapeutic target for heat-related illness and suggest that astaxanthin may potentially be an effective preventive strategy.

## KEYWORDS

antioxidant, cell injury, heat stress, mitochondria, reactive oxygen species

## 1 | INTRODUCTION

Under environmental conditions of extreme heat, internal heat production coupled with the environmental heat load can result in

insufficient heat dissipation, overall thermoregulatory failure, and thus overt hyperthermia (Sawka, Leon, Montain, & Sonna, 2011). The centers for disease control and prevention estimates that extreme heat kills more people in the United States than all other extreme weather events

combined. Heat-related illnesses thus pose a threat to public health, and frequent heat waves significantly impact human health, productivity, and the economy (Kjellstrom et al., 2016). As of today, around 30% of the world's population is exposed to deadly heat waves for at least 20 days during the year; this number may climb above 70% by the end of the century (Mora et al., 2017). New approaches for mitigating or minimizing heat stress-related injuries are clearly needed.

Muscle cramping and pain are among the first symptoms of heat-related illness (Becker & Stewart, 2011; Nelson, Collins, Comstock, & McKenzie, 2011), thus skeletal muscle should be considered a primary target for mitigating heat stress. In adult mouse skeletal muscle and C2C12 myoblasts, heat exposure initiates a cascade of events that can induce apoptosis (Yu, Deuster, & Chen, 2016; Yu et al., 2018). Also, heat stress stimulates the production of reactive oxygen species (ROS) that can exceed cellular antioxidant capacity, which is believed to contribute to the onset of heat-related skeletal muscle injuries (Davidson, Whyte, Bissinger, & Schiestl, 1996; Montilla et al., 2014; Yu et al., 2016, 2018). The precise mechanisms of heat-related injuries, however, remain to be elucidated.

Mitochondria are both the primary source and target of ROS (Galluzzi, Kepp, Trojel-Hansen, & Kroemer, 2012; Yoon, Galloway, Jhun, & Yu, 2011; Youle & van der Bliek, 2012). Accumulation of ROS in mitochondria induces mitochondrial outer membrane permeabilization (MOMP) and the release of proapoptotic proteins, such as cytochrome *c*, into the cytosol, which can then lead to the cleavage of several caspases and subsequent activation of the mitochondrial-dependent intrinsic apoptotic pathway (Katschinski, Boos, Schindler, & Fandrey, 2000; Wang et al., 2013). Heat stress also causes aberrant mitochondrial morphology, impaired mitochondrial function, and increased mitochondrial ROS production in yeast cells (Lewandowska, Gierszewska, Marszalek, & Liberek, 2006), rat cardiomyocytes (Qian, Song, Ren, Gong, & Cheng, 2004), mouse embryonic fibroblasts (Sanjuán Szklarz & Scorrano, 2012), mouse C2C12 myoblasts (Yu et al., 2016), and mouse skeletal muscle (Yu et al., 2018). Importantly, we have found that maintaining mitochondrial health prevents heat-induced ROS production and cell injury (Yu et al., 2016, 2018). These previously reported data suggest that mitochondria could be a therapeutic target for preventing heat illness and injury.

Astaxanthin is a red-orange carotenoid found in many aquatic organisms (Ambati, Phang, Ravi, & Aswathanarayana, 2014), and quercetin is a plant flavonoid present in fruits and vegetables (D'Andrea, 2015). Both astaxanthin and quercetin are used as ingredients in dietary supplements because of their recognized antioxidant properties (Brown, Gough, Deb, Sparks, & McNaughton, 2017; Cui, Li, Chang, Cong, & Hao, 2017; Ruiz et al., 2015). Preclinical studies and clinical trials have demonstrated beneficial effects of astaxanthin and quercetin supplementations on many human diseases due to oxidative stress, such as cancer, obesity, diabetes, cardiovascular diseases, and inflammatory and neurodegenerative diseases (Ambati et al., 2014; D'Andrea, 2015). This led to our hypothesis that both compounds could ameliorate

heat-induced ROS production and skeletal muscle injury. To explore this possibility, we examined mitochondrial integrity and function in cultured mouse C2C12 myoblasts and adult single myofibers isolated from the rat flexor digitorum brevis (FDB) following exposure to thermoneutral and heat stress conditions. A novel finding gleaned from this study is that of the two compounds tested, only astaxanthin preserves mitochondrial function and ameliorates heat-induced oxidative stress and skeletal muscle injury.

## 2 | MATERIALS AND METHODS

### 2.1 | C2C12 cell culture and heat-shock treatment

The mouse myoblast C2C12 cell line (ATCC<sup>®</sup> CRL-1772<sup>™</sup>) was cultured at 37°C in a 5% CO<sub>2</sub> humidified incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were passaged every ~2–3 days to maintain confluency below 60% to avoid differentiation. For heat-shock treatment, cells were placed in a CO<sub>2</sub> incubator with temperature preset at 43°C for the indicated time.

### 2.2 | Isolation of rat FDB myofibers

All procedures involving animals were approved by the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee. FDB myofibers isolation procedure was described previously (Dohl et al., 2018). Basically, Sprague–Dawley rats (Charles River, Germantown, MD) aged 20–24 weeks were euthanized by using isoflurane. The FDB muscle from both hind feet were harvested and incubated in 2 ml of DMEM containing 5.6 mM D-glucose, 0.2% bovine serum albumin and 0.1% gentamycin (wt/vol), collagenase (3 mg/ml) and dispase (1 mg/ml) at 37°C in a 5% CO<sub>2</sub> humidified incubator for ~2–3 hr. The muscles were then rinsed twice with fresh DMEM without collagenase and dispase, and gently triturated using glass pipette tips to free single myofibers. Isolated myofibers were transferred to dishes containing the same DMEM solution without collagenase and dispase and allowed to recover at 37°C in a 5% CO<sub>2</sub> humidified incubator for 1 hr. All of the myofibers were used for experiments on the same day of FDB harvest and isolation.

### 2.3 | Astaxanthin and quercetin treatment

Astaxanthin and quercetin were both purchased from Sigma-Aldrich (Burlington, MA). Stock solutions were made by dissolving astaxanthin (20 mM) or quercetin (100 mM) in dimethyl sulfoxide (DMSO); they were then stored at –20°C. On the day of treatment, stock solutions were further diluted in DMSO then directly added to the cells at 1:1,000 so the cell culture medium contained the desired concentrations of astaxanthin or quercetin. Astaxanthin or quercetin was added to the cells 30 min before heat stress, and maintained in the medium during the heat stress protocol. Control cells were treated with 0.1% DMSO only.

## 2.4 | Cell viability, caspase 3/7 activities, and cell death assays

Cell viability was determined by the trypan blue exclusion test with a Bio-Rad (Hercules, CA) TC20 automated cell counter per manufacturer's instruction, and confirmed by counting live cells under a light microscope. Caspase 3/7 activities were measured by CellEvent™ caspase-3/7 Green detection reagent (Invitrogen, Waltham, MA) following the manufacturer's instruction. Apoptosis was determined by using an Annexin V Alexa Fluor® 488 apoptosis kit (Invitrogen). Basically, cells grown on coverslips were incubated with annexin V (5/100 µl annexin-binding buffer) at room temperature for 15 min and washed twice in PBS. Annexin V positive (apoptotic) cells were then counted under fluorescence microscopy.

## 2.5 | Measurement of ROS levels and mitochondrial membrane potential

ROS levels were measured by using the fluorescent probe dihydroethidium (DHE) or the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA), per manufacturer's instructions. Mitochondrial membrane potential was evaluated by using the cationic fluorescent dye tetramethylrhodamine ethyl ester (TMRE). Basically, cells were incubated with 5 µM DHE, 10 mM H<sub>2</sub>DCFDA, or 100 nM TMRE in cell culture medium inside the CO<sub>2</sub> incubator for 30 min, then rinsed twice in warm DMEM. Fluorescence images were acquired at room temperature and saved for further analysis. DHE, H<sub>2</sub>DCFDA, and TMRE were all purchased from Invitrogen.

## 2.6 | Mitochondrial morphology analysis

Mitochondria were visualized by MitoTracker red CMXRos (Invitrogen) staining (100 nM in cell culture medium for 10 min). Images were acquired by a digital camera and saved for further analysis. The saved images were the adjusted using the ImageJ software (NIH, Bethesda, MD), and mitochondrial morphology quantitative analysis was performed.

## 2.7 | Fluorescence microscopy

Fluorescence images were viewed and acquired with a Nikon Eclipse Ti epifluorescence microscope equipped with a sCMOS pco.edge 4.2 digital camera (PCO AG, Kelheim, Germany) at room temperature. Excitation/emission wavelengths were 480/535 nm for annexin V and H<sub>2</sub>DCFDA, and 555/613 nm for MitoTracker red, TMRE, and DHE. All images were further modified and analyzed utilizing the ImageJ software (NIH).

## 2.8 | Cytosolic fractionation

Cytosolic fractionation was performed as described previously (Yu et al., 2014). Briefly, C2C12 cells were suspended in cold isolation buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] pH 7.2, 1 mM ethylenediaminetetraacetic acid, 320 mM

sucrose) containing protease inhibitors and homogenized in a Dounce homogenizer. The homogenate was centrifuged at 700g for 8 min. The first supernatant was saved, but the pellet was homogenized and centrifuged again. The two supernatants were pooled and centrifuged together at 17,000g for 15 min to obtain the mitochondrial pellet, the supernatant was kept as the cytosolic fraction.

## 2.9 | Western blot analysis

From cell lysates, 10 µg of protein was loaded to 4%–15% precast polyacrylamide gels (Bio-Rad), and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis. Western blot analysis was performed using the following primary antibodies: mouse anti-DLP1 (BD Biosciences, San Jose, CA), mouse anti-OPA1 (BD Biosciences), rabbit anti-GAPDH (Cell Signaling Technology, Beverly, MA), rabbit polyclonal anti-peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α; Abcam, Cambridge, MA), rabbit anti-mitochondrial transcription factor A (TFAM; Cell Signaling Technology), rabbit anti-cytochrome c (Cell Signaling Technology), rabbit anti-VDAC (Cell Signaling Technology), and mouse anti-actin (Santa Cruz Biotechnology, Dallas, TX). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were used as secondary antibodies. All the primary antibodies were used at 1:1,000 dilution except actin (1:2,000) and secondaries were used at 1:5,000. The bands were visualized with western ECL blotting substrates (Bio-Rad) and images were acquired using a Bio-Rad ChemiDoc MP Imaging System (Bio-Rad). Image densitometry was analyzed using ImageJ (NIH).

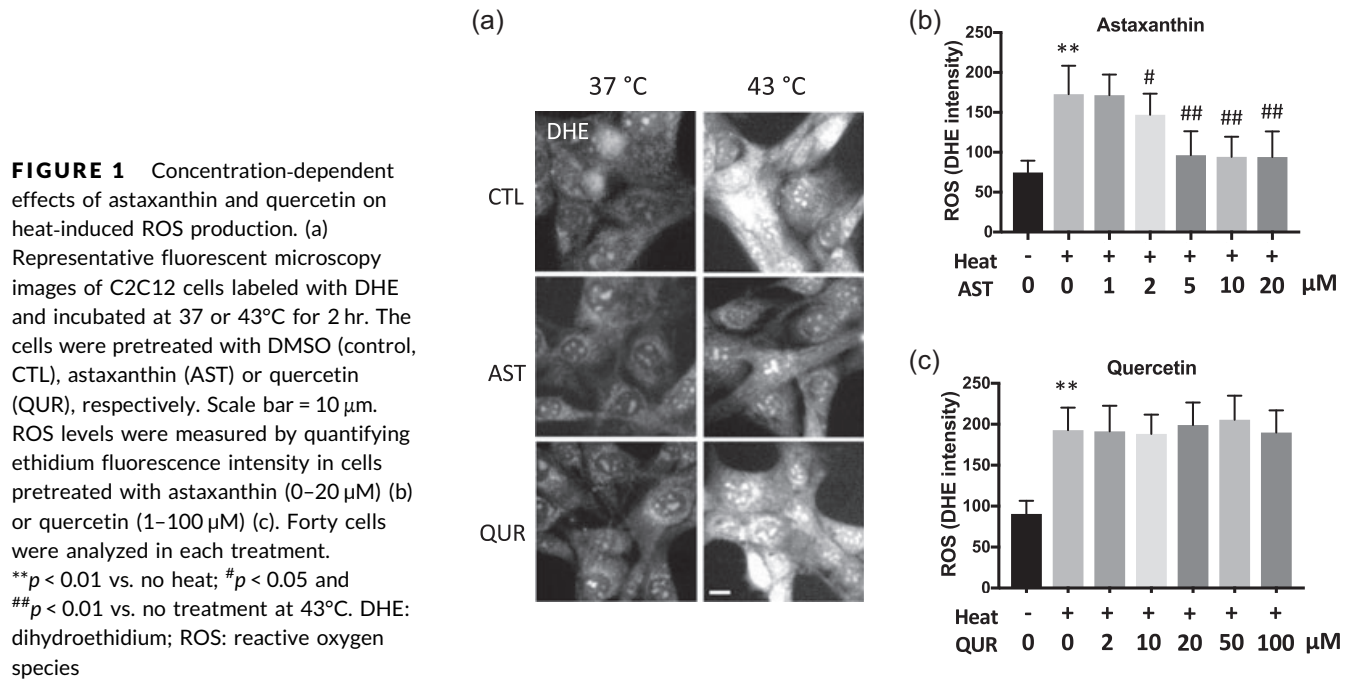
## 2.10 | Statistical analysis

All experiments were repeated independently a minimum of three times. A one-way analysis of variance was used to determine differences between groups. All data are presented as means ± SD. Values of  $p < 0.05$  were considered statistically significant.

# 3 | RESULTS

## 3.1 | Concentration-dependent effects of astaxanthin and quercetin on heat-induced ROS production in C2C12 myoblasts

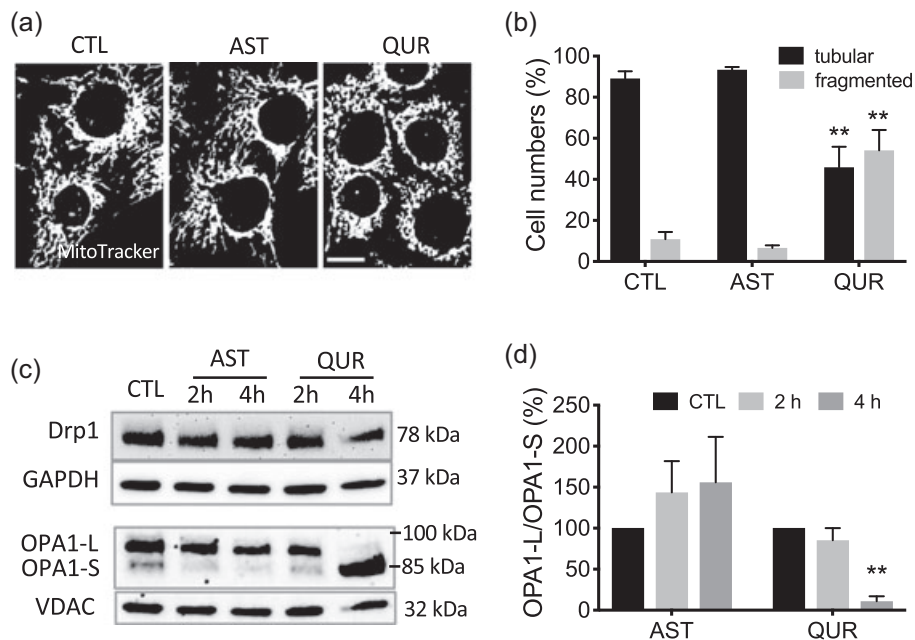
We previously reported that heat exposure at 43°C decreased cell viability in C2C12 myoblasts in a time-dependent manner: the survival rates were ~80% and 60% following 2 and 4 hr of 43°C heat stress, respectively (Yu et al., 2016); only <20% cells survived at 6 hr of heat exposure. Therefore, we exposure C2C12 myoblasts to 43°C heat stress for 2 and/or 4 hr in this study. We first examined whether astaxanthin and quercetin could prevent heat-induced ROS production. Cellular ROS levels were measured by ethidium fluorescence resulting from the oxidation of DHE (Figure 1a). Two hours heat exposure significantly increased ROS levels in C2C12 cells, consistent with our previous studies (Yu et al., 2016, 2018). Astaxanthin treatment inhibited heat-induced



ROS production in a concentration-dependent manner: showing mild protection at a concentration of 2  $\mu$ M and peaking at 5  $\mu$ M (Figure 1b). Treating C2C12 cells with quercetin did not prevent heat-induced ROS production, with levels remaining elevated at concentrations ranging from 2 to 100  $\mu$ M (Figure 1c).

### 3.2 | Effects of astaxanthin and quercetin on mitochondrial morphology in C2C12 myoblasts

Since mitochondria are a major source of ROS in mammalian cells, we labeled C2C12 cells with MitoTracker Red and examined the effects of astaxanthin and quercetin on mitochondrial morphology. Unexpectedly,



**FIGURE 2** Effects of astaxanthin and quercetin on mitochondrial morphology at 37°C. (a) Representative fluorescent microscopy images of C2C12 cells labeled with MitoTracker Red and treated with DMSO (CTL), astaxanthin (AST), or quercetin (QR) for 4 hr. Scale bar = 10  $\mu$ m. (b) Quantification of the cells containing tubular and fragmented mitochondria. Approximately 400 cells were counted in each treatment, \*\* $p$  < 0.01 vs. CTL. (c) Representative western blot images of mitochondrial fission protein Drp1 and fusion protein OPA1 in C2C12 cells. GAPDH was used as a loading control for Drp1, and VDAC for OPA1. L and S represent long- and short-form of OPA1, respectively. (d) Densitometric analysis of OPA1 protein long- to short-form ratio in C2C12 cells. \*\* $p$  < 0.01 vs. CTL. DMSO: dimethylsulfoxide; GAPDH: glyceraldehyde 3-phosphate dehydrogenase

under normal culture temperature (37°C), 54.1% of cells treated with quercetin (10  $\mu$ M) contained smaller, shorter, and fragmented mitochondria, compared with only 10.9% of control cells with fragmented mitochondria (Figure 2a,b). In contrast, 93.4% of cells treated with astaxanthin (5  $\mu$ M) maintained tubular mitochondrial structure and were slightly elongated (Figure 2a). Consistent with the mitochondrial morphology data, quercetin treatment of 4 hr induced cleavage of the mitochondrial inner-membrane fusion protein OPA1 from its long- to short-form, which resulted in a significant lower OPA1 long- to short-form ratio (Figure 2c,d). Astaxanthin treatment increased the OPA1 long- to short-form ratio, but the difference was not statistically significant. Both astaxanthin and quercetin treatments had no significant impact on mitochondrial fission protein Drp1 expression at 37°C (Figure 2c).

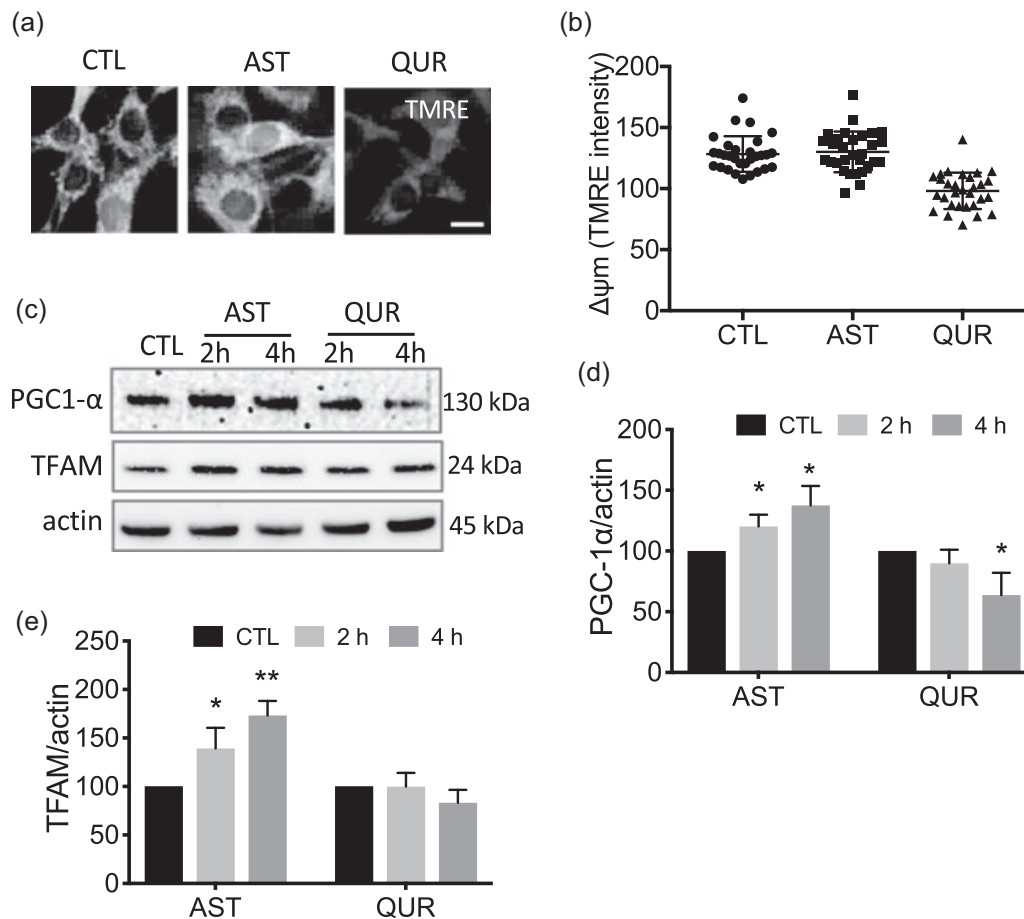
### 3.3 | Effects of astaxanthin and quercetin on mitochondrial functions in C2C12 myoblasts

To assess mitochondrial function, we stained C2C12 cells with TMRE and analyzed mitochondrial membrane potential ( $\Delta\Psi_m$ ). Under normal

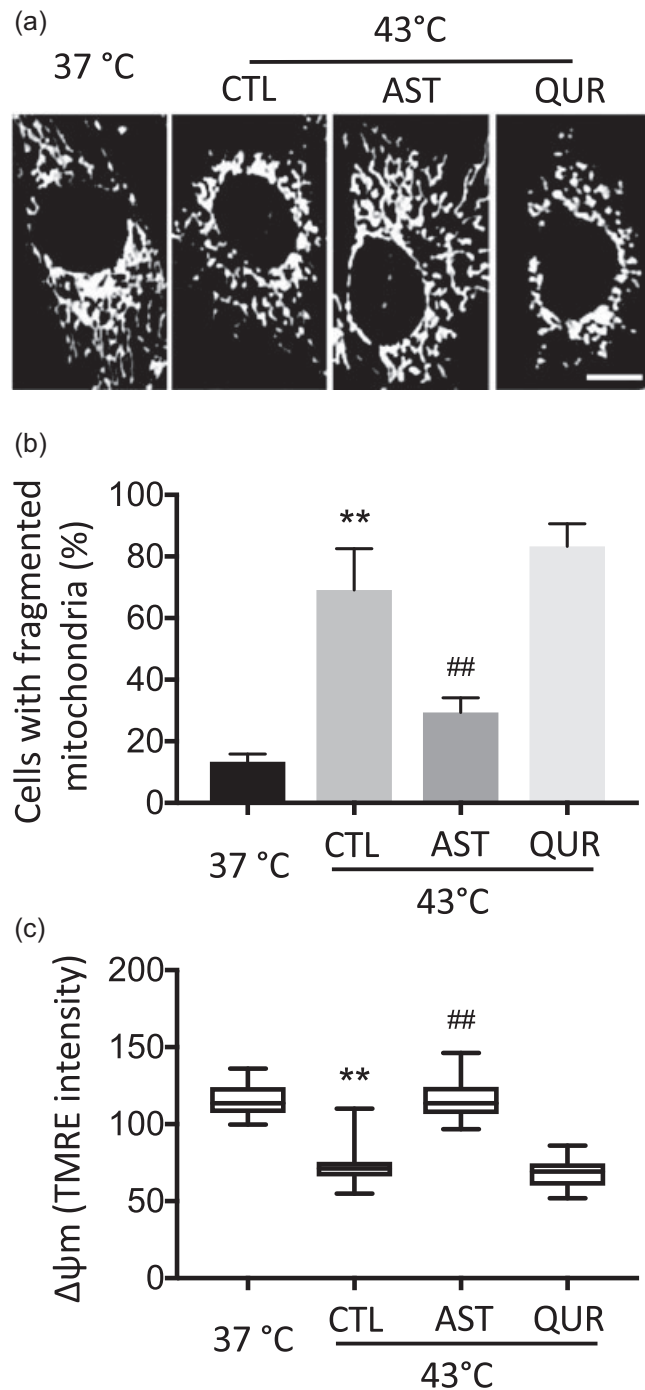
culture temperature (37°C), astaxanthin treatment maintained  $\Delta\Psi_m$  similar to that of control cells (Figure 3a,b). Quercetin, however, decreased  $\Delta\Psi_m$  (Figure 3a,b). The expression of PGC-1 $\alpha$ , the master regulator of mitochondrial biogenesis, and TFAM, a key activator of mitochondrial DNA (mtDNA) replication (Fernandez-Marcos & Auwerx, 2011; Uchiyama & Kang, 2012), were increased in cells treated with astaxanthin in a time-dependent manner (Figure 3c–e). In contrast, quercetin had no effect on TFAM, but after 4 hr of treatment expression of PGC-1 $\alpha$  was significantly suppressed (Figure 3c–e).

### 3.4 | Astaxanthin, but not quercetin, prevents heat-induced mitochondrial fragmentation and decreased $\Delta\Psi_m$ in C2C12 myoblasts

Our previous studies showed that heat stress caused mitochondrial deformation and dysfunction (Yu et al., 2016, 2018). We next explored whether astaxanthin and quercetin alter mitochondrial morphology and function under heat stress conditions. Exposing C2C12 myoblasts to 43°C resulted in mitochondrial fragmentation and decreased  $\Delta\Psi_m$  (Figure 4a–c). In myoblasts treated with



**FIGURE 3** Effects of astaxanthin and quercetin on mitochondrial function at 37°C. (a) Representative fluorescent microscopy images of C2C12 cells stained with TMRE and treated with DMSO (CTL), astaxanthin (AST, 5  $\mu$ M), or quercetin (QUR, 10  $\mu$ M). Scale bar = 10  $\mu$ m. (b) Quantification of TMRE fluorescence intensity. Forty cells were analyzed in each treatment. (c–e) Representative western blot images and densitometric analysis of PGC-1 $\alpha$  and TFAM protein levels in C2C12 cells. \* $p < 0.05$  and \*\* $p < 0.01$  vs. CTL. DMSO: dimethylsulfoxide; PGC-1 $\alpha$ : peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$ ; TFAM: mitochondrial transcription factor A; TMRE: tetramethylrhodamine ethyl ester



**FIGURE 4** Astaxanthin, but not quercetin, prevents heat-induced mitochondrial fragmentation and decreased  $\Delta\Psi_m$ . (a) Representative fluorescent microscopy images showing mitochondrial morphology in C2C12 cells treated with DMSO (CTL), astaxanthin (AST, 5  $\mu\text{M}$ ), or quercetin (QUR, 10  $\mu\text{M}$ ) at 37 or 43°C, respectively. Scale bar = 10  $\mu\text{m}$ . (b) Quantification of cells containing fragmented mitochondria. Approximately 400 cells were counted in each treatment. (c) Quantification of  $\Delta\Psi_m$  (TMRE fluorescence intensities). Forty cells were analyzed in each treatment. \*\* $p < 0.01$  vs. 37°C; ## $p < 0.01$  vs. CTL. DMSO: dimethylsulfoxide; TMRE: tetramethylrhodamine ethyl ester

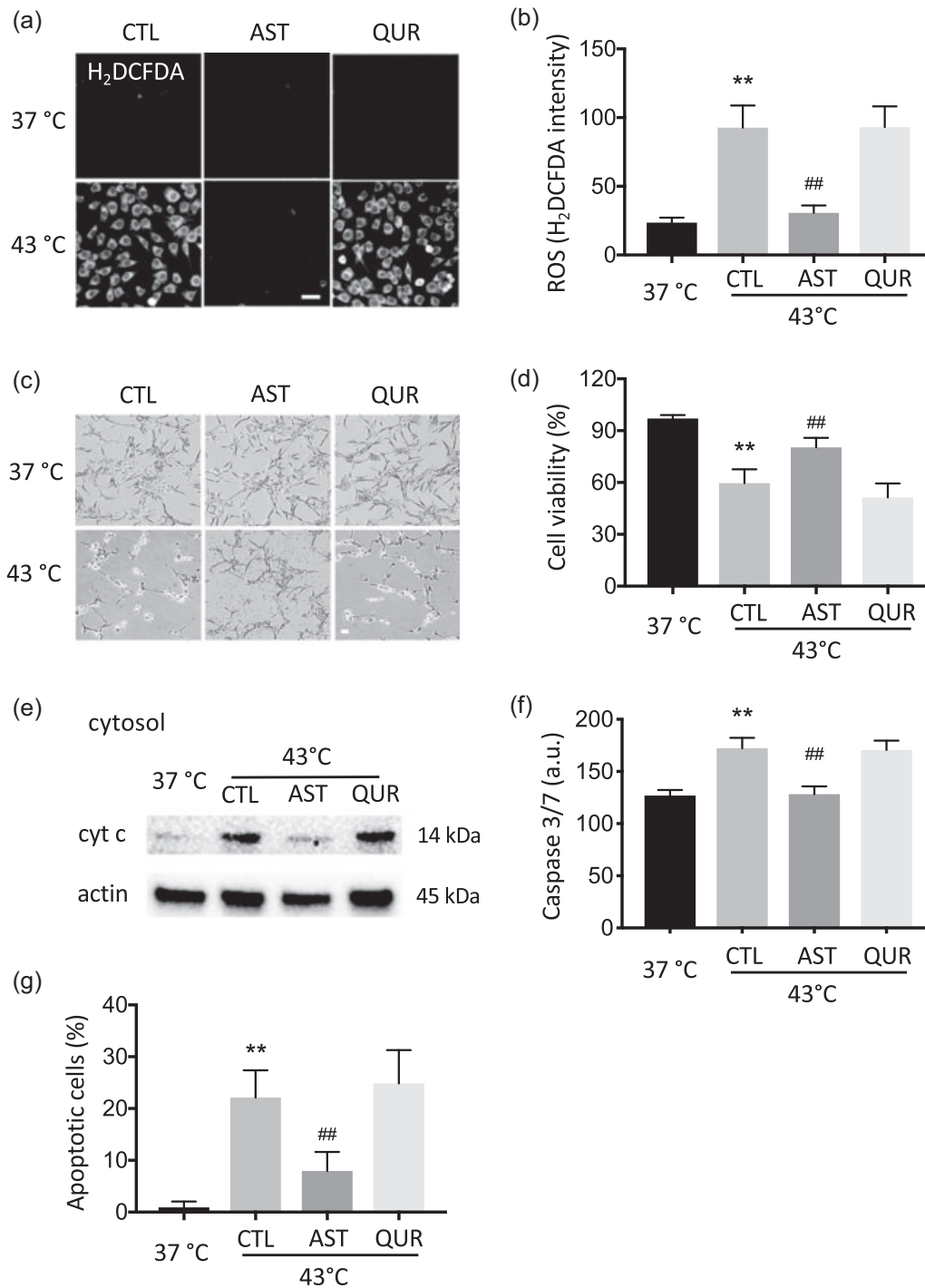
astaxanthin (5  $\mu\text{M}$ ) and exposed to 43°C, the mitochondrial tubular network and  $\Delta\Psi_m$  were preserved similar to cells incubated at 37°C (Figure 4a–c). In contrast, mitochondria in quercetin-treated cells (10  $\mu\text{M}$ ) were excessively fragmented and swollen at 43°C (Figure 4a,b).  $\Delta\Psi_m$  was low in both control and quercetin-treated cells at 43°C, whereas astaxanthin treatment preserved  $\Delta\Psi_m$  in cells incubated at 43°C at a level similar to the control cells at 37°C (Figure 4c).

### 3.5 | Astaxanthin, but not quercetin, prevents heat-induced ROS production, and cell death in C2C12 myoblasts

Heat exposure at 43°C mainly induces apoptosis, whereas >45°C causes necrosis (Harmon et al., 1990; Yu et al., 2016). Due to the purported contribution of ROS to heat-induced muscle injury, we examined whether astaxanthin or quercetin could prevent heat-induced apoptosis in C2C12 myoblasts exposure to 43°C heat stress. First, we used  $\text{H}_2\text{DCFDA}$ , another fluorescent probe, and confirmed that astaxanthin inhibited heat-induced ROS production, whereas quercetin did not (Figure 5a,b). Heat exposure (43°C) induced cell detachment (Figure 5c) and significantly decreased cell viability (Figure 5d). In addition, 43°C heat exposure released cytochrome c from the mitochondria into the cytosol (Figure 5e), activated caspase 3/7 (Figure 5f), and increased number of annexin V positive cells (Figure 5g), which suggests that heat-induced cell death includes apoptosis. Astaxanthin treatment not only normalized ROS levels (Figure 5a,b) under exposure to 43°C, but also prevented cell detachment, maintained cell viability and attenuated apoptosis (Figure 5c–g). Quercetin failed to protect cells from heat-induced injury and the results were similar to control cells under heat-stress conditions (Figure 5c–g).

### 3.6 | Astaxanthin but not quercetin, prevents heat-induced mitochondrial deformation, ROS production, and injury in rat FBD myofibers

To confirm the results obtained from C2C12 myoblasts, we isolated rat FBD myofibers and incubated them at 37 or 43°C. As depicted in Figure 6a, mitochondria in myofibers treated with astaxanthin and incubated at 43°C remained well organized and showed distinct rings evenly distributed throughout the fibers, similar to myofibers exposed to 37°C. In contrast, mitochondria in myofibers with sham or quercetin treatment became disorganized and more irregular at 43°C. ROS production was also significantly higher at 43°C in myofibers with sham or quercetin treatment compared with that at 37°C, yet it remained low in myofibers treated with astaxanthin (Figure 6b). Moreover, 62% and 79% of fibers with sham or quercetin treatment were hypercontracted at 43°C, compared with 8% of hypercontracted fibers at 37°C. This number fell to 29% in myofibers treated with astaxanthin (Figure 6c). Hypercontraction represents a common mechanism



**FIGURE 5** Astaxanthin, but not quercetin, prevents heat-induced ROS production and cell death. These cells were pretreated with DMSO (CTL), astaxanthin (AST, 5  $\mu$ M), or quercetin (QUR, 10  $\mu$ M), and incubated at 37 or 43°C, respectively. (a) Representative fluorescent microscopy images of C2C12 cells loaded with H<sub>2</sub>DCFDA. (b) ROS levels were measured by quantifying H<sub>2</sub>DCFDA fluorescence intensity. (c) Representative bright field images of C2C12 cells. (d) Quantification of cell viability. (e) Representative images of cytochrome c (cyt c) western blot analysis in the cytosolic fractions of C2C12 cells. (f) Quantification of caspase 3/7 activities. (g) Quantification of annexin V positive (apoptotic) cells. Forty cells were analyzed in each treatment. \*\* $p < 0.01$  vs. 37°C; ## $p < 0.01$  vs. CTL. Scale bar = 20  $\mu$ m. DMSO: dimethylsulfoxide; H<sub>2</sub>DCFDA: 2',7'-dichlorodihydrofluoresceindiacetate; ROS: reactive oxygen species

for muscle damage (Montana & Littleton, 2006). Taken together, these results suggest that astaxanthin, but not quercetin, prevents heat-induced mitochondrial deformation, ROS production, and skeletal muscle injury.

#### 4 | DISCUSSION

We have previously reported that heat stress induces mitochondrial fragmentation and production of ROS, which leads to oxidative

damages in mouse skeletal muscle and C2C12 mouse myoblasts (Yu et al., 2016, 2018). Oxidative stress has been suggested to contribute to the pathogenesis of heat-related illnesses (Davidson & Schiestl, 2001; Wang et al., 2013). In the present study, we tested two dietary antioxidants—astaxanthin and quercetin—in an effort to identify countermeasures to ameliorate heat stress-induced oxidative damage in cultured C2C12 myoblasts and adult rat myofibers. Our results indicate that astaxanthin, but not quercetin, can prevent heat stress-induced ROS production, cell injury, and skeletal muscle damage.

The disparate results observed with astaxanthin and quercetin in heat stress-induced oxidative injury appears to stem in part from their effects on mitochondrial morphology and function. Astaxanthin promoted mitochondrial biogenesis and prevented heat-induced mitochondrial fragmentation, which resulted in decreased ROS production and cell injury at 43°C. In contrast, quercetin caused mitochondrial fragmentation, suppressed mitochondrial biogenesis, and decreased membrane potential at 37°C, and failed to protect against heat-induced mitochondrial deformation/dysfunction and oxidative injuries. Our results further confirm a critical role of mitochondria in oxidative injury induced by heat stress.

Lethal heat shock is known to directly impair the mitochondrial electron transport chain (ETC) and cause mitochondrial dysfunction (Davidson & Schiestl, 2001; White et al., 2012). The ETC is embedded in the inner mitochondrial membrane. Heat stress also causes excessive mitochondrial fragmentation (Sanjuán Szklarz & Scorrano, 2012; Yu et al., 2016, 2018), which then may disrupt ETC structure and function. Impairment of mitochondrial ETC is known to lead to increased production of ROS (Zorov, Juhaszova, & Sollott, 2014), generated as an inevitable byproducts of oxidative phosphorylation in the ETC (Yu, Wang, & Yoon, 2015). Accumulation of ROS within mitochondria can then directly oxidize components of the ETC, including lipids, proteins, and mtDNA, which in turn results in mitochondrial dysfunction and energy depletion (Lee, Giordano, & Zhang, 2012). In addition, under thermal stress, free radicals induce MOMP and release proapoptotic proteins such as cytochrome c into the cytosol, which leads to downstream caspase activation and subsequent apoptosis (Katschinski et al., 2000; Wang et al., 2013).

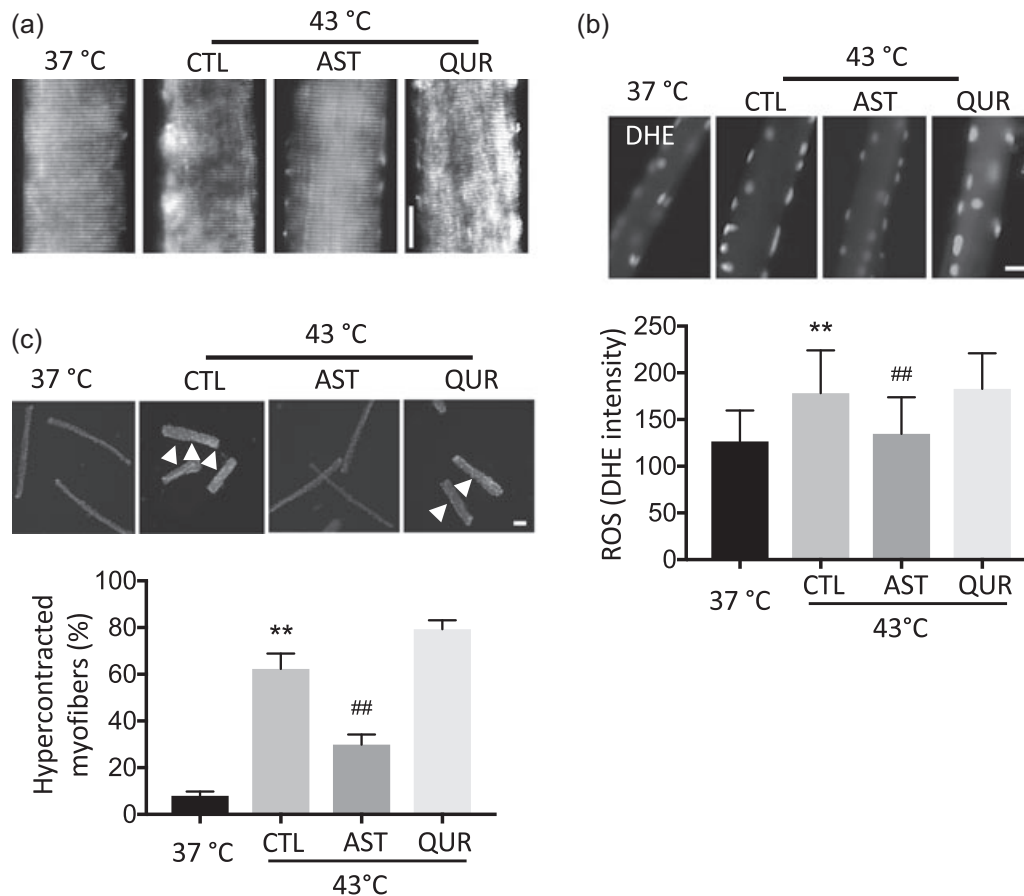
Astaxanthin has the highest antioxidant activity among all the natural sources of carotenoids, about 100 times stronger than vitamin E (Ambati et al., 2014; Kurashige, Okimasu, Inoue, & Utsumi, 1990). As an antioxidant, astaxanthin donates electrons to free radicals to reduce them into more stable forms (Ambati et al., 2014). In addition, astaxanthin increases the activity of endogenous antioxidant enzymes including superoxide dismutase, catalase, and glutathione (Ambati et al., 2014; Brown et al., 2017). Astaxanthin is lipophilic, thus it accumulates within the mitochondrial membrane and regulates the mitochondrial redox state (Liu, Shibata, Hisaka, & Osawa, 2009; Wolf et al., 2010). In the present study, the increased expression of PGC-1 $\alpha$  and TFAM observed in myoblasts treated with astaxanthin at 37°C suggests that mitochondrial biogenesis occurred. Also, astaxanthin maintained mitochondrial structure and function under heat-stress. Thus, our data support an indirect antioxidant

benefit of astaxanthin, that is, it prevents heat-induced ROS production and cell injury by preserving mitochondrial integrity.

Quercetin, a plant flavonoid present in fruits and vegetables, has been reported to exert powerful antioxidant effects in several different animal and cellular models of human disease (Boots, Haenen, & Bast, 2008; D'Andrea, 2015). Like astaxanthin, quercetin interacts directly with mitochondria and regulates their function (de Oliveira et al., 2016). However, recent studies have raised some controversy by suggesting that quercetin can have a pro-oxidant effect both in vitro and in vivo, depending on the concentration and the cellular redox status (Boots et al., 2007; Fonseca-Silva, Inacio, Canto-Cavalheiro, & Almeida-Amaral, 2011; Ruiz et al., 2015; Xia et al., 2015). Treatment with quercetin in mice impaired mitochondrial ETC and caused a decrease in the protein expression of mitochondrial outer protein Mfn2 (Ruiz et al., 2015). In isolated rat liver mitochondria, quercetin interacted with the mitochondrial membrane and significantly inhibited respiration (Dorta et al., 2008, 2005). Quercetin also induces production of ROS and the mitochondrial permeability transition in isolated mitochondria and cultured cells (De Marchi, Biasutto, Garbisa, Toninello, & Zoratti, 2009). However, our results do not support a role for quercetin in promoting ROS production in excess of what occurs with heat stress in myoblasts or adult myofibers.

As discussed above, astaxanthin and quercetin are both lipophilic and can interact with mitochondria directly (de Oliveira et al., 2016; Liu et al., 2009; Wolf et al., 2010). It is unlikely, the diverse effects of astaxanthin and quercetin on heat shock response are due to their different lipophilicities. The molecular mechanism as to how astaxanthin, but not quercetin, prevents heat-induced changes in mitochondrial morphology and function is not clear in the present study and remains to be further investigated. We and others have previously reported that heat exposure caused activation of mitochondrial fission protein Drp1 and cleavage of fusion protein OPA1, which shifts mitochondrial fission–fusion balance toward fission, and thereby would contribute to heat-induced mitochondrial fragmentation (Sanjuán Szklarz & Scorrano, 2012; Yu et al., 2016, 2018). Electron micrographs of mouse skeletal muscle showed that fragmented mitochondria in heat-shocked muscle more frequently displayed lower cristae density and disorganized structure, which reflect mitochondrial dysfunction (Yu et al., 2016, 2018). Importantly, inhibition of fission not only restored all the changes in mitochondrial ultrastructure, but also prevented heat-induced mitochondrial dysfunction and cell injury (Yu et al., 2016, 2018). Therefore, astaxanthin and quercetin may have different effects in mitochondrial fission and fusion machinery under heat stress conditions, which could contribute to their diverse effects on heat shock response.

In the present study, we found that quercetin suppressed the expression of PGC-1 $\alpha$ , and promoted the cleavage of OPA1 from its long form into its short form (Figures 2c, and 3c,d). OPA1 mediate fusion of the inner mitochondrial membrane and maintain cristae structure (Yu et al., 2015). OPA1 long-form cleavage infers abnormal fusion and is associated with mitochondrial dysfunction (Ishihara, Fujita, Oka, & Mihara, 2006; Song, Chen, Fiket, Alexander, & Chan, 2007). The data presented herein implies that quercetin inhibits mitochondrial fusion machinery, shifting the mitochondrial fission–fusion balance toward



**FIGURE 6** Astaxanthin, but not quercetin, prevents heat-induced mitochondrial deformation, ROS production and injury in rat FDB myofibers. (a) Rat FDB myofibers were labeled with MitoTracker Red and incubated at 37 or 43°C for 30 min. The fibers were pretreated with DMSO (CTL), astaxanthin (AST, 5  $\mu$ M), or quercetin (QUR, 10  $\mu$ M), respectively. (b) Representative fluorescent microscopy images and measurement of DHE staining. Forty fibers were analyzed in each treatment. (c) Representative images of hypercontracted (arrowhead) myofibers and quantitative analysis. Twenty fields ( $\times 10$ ) were randomly selected in each treatment. \*\* $p < 0.01$  vs. 37°C; ## $p < 0.01$  vs. CTL. Scale bar = 10  $\mu$ m. DHE: dihydroethidium; DMSO: dimethylsulfoxide; FDB: flexor digitorum brevis; ROS: reactive oxygen species

fission. As a result, quercetin treatment caused mitochondrial fragmentation and decreased membrane potential at normal incubation temperatures. Moreover, quercetin treatment failed to prevent both heat stress-induced mitochondrial deformation and dysfunction and all of the cellular events leading to oxidative damage under heat stress conditions.

Several limitations must be recognized. First, various forms of quercetin exist and responses to their administration differ. For example, unlike quercetin, dihydroquercetin does not inhibit stress-induced heat shock protein expression (Budagova, Zhmaeva, Grigor'ev, Goncharova, & Kabakov, 2003). Moreover, dihydroquercetin, otherwise known as "Taxifolin" has a number of reported beneficial effects on oxidative stress and cell apoptosis (Sun et al., 2014; Vladimirov et al., 2009; Zai et al., 2018), which may not be consistent with the effects of quercetin. Thus, the form might be important and we studied only quercetin. Second, doses of astaxanthin vary widely—and may range from 500 to 6.0 g/day—cell culture doses are likely different from in vivo studies and doses between 1 and 100 mg/kg or up to ~600 mg have been used. Human doses have ranged from a low of 0.2 mg/day up to 100 mg/day (Ambati et al., 2014). Other limitations include the fact that these data cannot be translated into animal or even human studies.

So, despite the interesting results, additional work will be necessary before translating into human studies.

In summary, we observed that astaxanthin and quercetin induced opposite changes in mitochondria morphology and function. These changes appear closely correlated with their antioxidant effects under heat stress conditions. Astaxanthin enhanced mitochondrial biogenesis and preserved mitochondrial integrity, thus protecting skeletal muscle against heat stress-induced oxidative damage. In contrast, quercetin inhibited mitochondrial fusion machinery and caused mitochondrial depolarization, ultimately failing to preserve mitochondrial function and prevent heat stress-induced cell injury. Our results confirm that mitochondria could be an effective therapy target and suggest that astaxanthin, but not quercetin, may be efficacious in preventing heat-related illness.

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## CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest. The views expressed are those of the authors and do not reflect the official position of the Uniformed Services University of Health Sciences, the United States Navy or the Department of Defense.

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