Journal of Molecular Neuroscience
https://doi.org/10.1007/s12031-018-1197-9

Mitochondrial Electron Transport Chain Complex Dysfunction in MeCP2 Knock-Down Astrocytes: Protective Effects of Quercetin Hydrate

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Received: 25 May 2018 / Accepted: 15 October 2018

Abstract

Astrocytes play the central role in CNS metabolism to support neuronal functions. Mehyl-CpG-binding protein 2 (MeCP2) is the global transcription factor with differential expression in neuronal and non-neuronal cells. MeCP2 mutation and downstream detrimental effects have been reported in astrocytes also in MeCP2-associated neurodevelopmental disorder-Rett syndrome. Several studies have shown mitochondrial impairment linked to ROS production and reduced ATP synthesis in Rett patients and models, but consequences of MeCP2 deficiency on mitochondrial electron transport chain complexes in astrocytes and effect of known antioxidant quercetin aglycone has not yet been reported. The present study aimed to investigate effect of quercetin on mitochondrial functioning in MeCP2-deficient astrocytes. Our data show onefold upregulated Uqcrc1 and Ndufv2 gene expression, subtle change in protein expression, and significantly reduced mitochondrial respiratory chain complex-II and complex-III enzyme activities in MeCP2 knock-down astrocytes. Intracellular calcium robustly increased and mitochondrial membrane potential decreased, while no change in ROS was observed in MeCP2 knock-down astrocytes. Quercetin increased MeCP2 and normalized Uqcrc1 and Ndufv2 gene expression but did not modulate MeCP2 and Ndufv2 proteins expression. Interestingly, quercetin upregulated significantly the mitochondrial respiratory complex-II, complex-III, and complex-IV activities in dose-dependent manner. It also restored intracellular calcium level and mitochondrial membrane potential. In vitro observations suggest the beneficial effect of quercetin in mitochondrial functioning in MeCP2-deficient condition. There are no reports focusing on role of quercetin in mitochondrial function in MeCP2-deficient astrocytes, and these observations serve as preliminary data to evaluate quercetin's effects in vivo.

Keywords MeCP2 · Electron transport chain · Mitochondria · Astrocytes · Quercetin aglycone

Abbreviations

MeCP2	Methyl-CpG-binding protein 2
RTT	Rett syndrome
NC	Negative control
S2	MeCP2 siRNA-treated
Uqcrc1	Ubiquinol cytochrome c reductase core protein
Ndufv2	NADH dehydrogenase (ubiquinone) flavoprotein 2
CNS	Central nervous system
MMP	Mitochondrial membrane potential
MRC	Mitochondrial respiratory chain
QH	Quercetin hydrate

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Introduction

MeCP2, the second Methyl CpG binding protein, was first identified in rat brain and kidney cells (Lewis et al. 1992) which was later on discovered to be mutated (loss-of-function) and associated with pathogenesis of Rett syndrome—an autism spectrum neurodevelopmental disorder (Amir et al. 1999). In CNS, neurons possess the highest expression of MeCP2, but recently, MeCP2 expression in glial cells has also been reported (Ballas et al. 2009; Derecki et al. 2012; Lioy et al. 2011; Maezawa and Jin 2010; Parikh et al. 2017; Sharma et al. 2015; Vora et al. 2010; Yasui et al. 2013; Nguyen et al. 2013; Maezawa et al. 2009; Buch et al. 2018). Although the MeCP2 expression in glial cells is low being lowest in astrocytes (Ballas et al. 2009), MeCP2 modulates gene expression pathways in astrocytes (Yasui et al. 2013).In addition, the evidences show astrocytes carrying MeCP2 mutations causing aberrant neuronal dendritic morphology (Ballas et al. 2009; Maezawa et al. 2009) and impaired myelin gene expression in

Published online: 06 December 2018

oligodendrocytes—dorsal root ganglion (DRG) neurons—astrocyte co-culture.

Several reports provide an insight into mitochondrial dysfunction in brain of MeCP2 null mouse and Rett syndrome patients reviewed in detail by Shulyakova et al. (2017). Altered mitochondrial respiratory chain complex genes, proteins, and enzyme activities, and reduced ATP level have also been documented in RTT condition (Kriaucionis et al. 2006; Pecorelli et al. 2013; Gold et al. 2014; Valenti et al. 2017; De Filippis et al. 2015; Li et al. 2013; Saywell et al. 2006; Gibson et al. 2010; Jin et al. 2015). It has been suggested that progressive deterioration of motor functions and brain dysfunction in RTT may be related to the ROS overproduction and that potentially contribute to RTT-like symptoms (De Filippis et al. 2015; Kriaucionis et al. 2006). Moreover, reversal of RTT clinical signs by antioxidants, such as ω-3 polyunsaturated fatty acids (De Felice et al. 2012), Trolox (Janc and Müller 2014), and reduced vitamin E levels (Formichi et al. 1998) and altered ROS-stabilizing enzymes such as superoxide dismutase, catalase, and glutathione S-transferase (Gold et al. 2014; Großer et al. 2012; Pecorelli et al. 2013) in RTT, indicates potential role of mitochondrial impairment in RTT pathogenesis.

Quercetin, a plant favonol, is a widely studied compound having neuroprotective role (Costa et al. 2016) and modulatory effects on intracellular signaling pathways and epigenetic mechanisms in vitro and in vivo (Cacabelos and Torrellas 2015; Williams et al. 2004). Quercetin is effective in improving mitochondrial dysfunctions in many of the diseases (Qiu et al. 2018; Sandhir and Mehrotra 2013; Carrasco-Pozo et al. 2012; Chakraborty et al. 2012; Yeganeh et al. 2018) and has also been found to modulate L-type calcium channel in pituitary tumor (GH3) cells and neuronal NG108-15 cells differentially that indicates the ability of quercetin in regulating ion channels dependent on cell type or calcium level (Wu et al. 2003). Brain-derived neurotrophic factor (BDNF) improves calcium regulation speculated to be mediated by SERCA, Ca2+ ATPase that transfers Ca2+ from the cytosol of the cell to the lumen in MeCP2-deficient neurons (Mironov et al. 2009). Interestingly, quercetin has been documented to upregulate (BDNF) in Alzheimer mouse model (Hou et al. 2010) and in acute hypobaric hypoxia (HH) condition (Liu et al. 2015). Hence, Increased BDNF expression following quercetin treatment might benefit in impaired calcium homeostasis.

In the present study, we thus explored whether mitochondrial respiratory chain complex genes, protein, and enzyme activities are altered in MeCP2-deficient astrocytes and whether these can be ameliorated by quercetin treatment. To this aim, we performed MeCP2 knock-down in astrocytes by siRNA transfection and observed effect of pre-treated quercetin (25 μM, 100 μM) in the same. We also tried to figure out whether Ca^{+2} homeostasis, ROS generation, and mitochondrial membrane potential too are affected as a consequence of mitochondrial respiratory chain (MRC) impairment and if quercetin can improve this. To the best of our knowledge, this is the first report focusing on quercetin's protective effect in MeCP2 knock-down astrocytes.

Materials and Methods

Primary Astrocytes Culture

Cortical astrocytes were isolated from Charles foster neonatal rat pups (post-natal day P0–2) by a well described method (Chen et al. 2007; Sharma et al. 2015). In brief, in the sterile conditions, cerebral cortex was isolated followed by the removal of meninges in ice cold 1× HBSS. These meninges-free cortices were diced and digested at 37 °C with 0.2 mg/ml DNase I and 0.25% TPVG in 1× HBSS. After attaining single cell suspension following centrifugation and passing through 70-μm nylon cell strainer, cells were plated in poly-l-lysine (PLL-10 μg/ml)-coated flasks with complete medium-DMEM (Gibco) containing high glucose, 4 mM L-glutamine supplemented with 10% FBS, and 1% penicillin and streptomycin, and then, flasks were incubated at 37 °C with 5% CO_2. Cells were fed with complete medium change every 2–3 days until the 10th day in vitro (DIV). At 10th DIV, mixed glial cultures were confluent; process-bearing OPCs appeared on top of phase-gray bed layers of astrocytes. Microglial cells were removed by shaking flask horizontally at 200 r.p.m. for 1 h followed by OPCs for overnight at 37 °C. After removing the cell suspension post-shaking, adherent pure astrocytes (98–100%) grown in monolayer were obtained. These astrocytes were sub-cultured on the day before the experiment in PLL-coated 12-well plates, 96-well plates, or coverslips for immunocytochemistry.

Treatments and siRNA Transfection

Cells were incubated with quercetin hydrate (Sigma, Cat. No. 337951) for 4 h followed by 20 nM MeCP2 siRNA (SASI_Rn01_00072926, Sigma) or universal negative siRNA (SIC001) (in negative control) transfection using HiPerFect reagent (Qiagen) for 24 h as per the manufacturer instructions.

Semi Quantitative and Quantitative Real-Time RT-PCR

Total RNA was harvested from astrocytes using trizol reagent (Invitrogen), and integrity-purity were checked by running RNA on 1% agarose gel in sterile condition. RNA was quantified by Qubit RNA assay kit in Qubit 2.0 fluorometer (Invitrogen), and 1 μg of total RNA was used to prepare cDNA using cDNA kit (Verso-Thermo Fisher, Cat. No. AB1453). A total of 1-μl cDNA was amplified by PCR—

35 cycles for Uqcrc1, Ndufv2, MeCP2, and housekeeping gene GAPDH. The PCR products were run on 2% agarose gel and visualized in gel doc (Bio-Rad). Band intensity was measured by Image J software (1.51j8) and normalized to GAPDH. Quantitative real-time PCR was performed in a QuantStudio 12K (Life Technology) real-time PCR instrument using SYBR-green PCR master mix (Applied Biosystems), and the data were analyzed by $2^{-\Delta\Delta CT}$ (Livak) method (Livak and Schmittgen 2001). Primers used for this analysis are as in Table 1.

Western Blot Analysis

Cell lysates were prepared in 2× Laemmli sample buffer. Total protein was quantified by Qubit protein assay kit in Qubit 2.0 fluorometer (Invitrogen) and 40 μg of total protein was resolved in 10% SDS-PAGE (100 V). Protein was transferred on 45-μm nitrocellulose membrane at constant voltage (100 V) for 90 min in cooling condition followed by blocking membrane with 3% BSA in TBST for 1 h with agitation. The membranes were probed with primary antibodies Ndufv-2 (1:250, Santa Cruz), MeCP2 (1:200,Santa Cruz), and β-actin (1:1000, Santa Cruz) for overnight at 4 °C followed by respective HRP-conjugated secondary antibodies (1:3000, Sigma) incubation at room temperature for 1 h with agitation. Chemiluminescence was captured in ChemiDoc (Fusion SL with VisionCapt v16.12 software), and the band intensities were measured by Image J software (1.51j8) and were normalized to internal control β-actin.

Immunocytochemistry

For the characterization and protein expression analysis, astrocytes grown on PLL-coated coverslips were fixed in 4% paraformaldehyde (15 min) in 1× PBS (pH 7.4) at room temperature. Cells were permeabilized by incubating with 0.25% tritonX-100 and blocked by 1% BSA in PBS-T (0.1% Tween 20). Primary antibody, i.e., glial fibrillary acidic protein (GFAP) antibody (1:500, pierce) diluted in blocking buffer was incubated for overnight at 4 °C in humidified chamber. In the dark, cells were incubated with respective FITC-labeled secondary antibody (1:500, Sigma) for 1 h at room temperature followed by nucleus staining with DAPI (0.1 μg/ml). Cover slips were mounted with 80% glycerol and sealed with nail polish. Slides were imaged in Carl Zeiss confocal microscope (× 60 magnification).

Mitochondrial Respiratory Chain (MRC) Enzymatic Activities (Spectrophotometric Methods)

For mitochondrial ETC enzyme activity assays, cell pellets were suspended in potassium phosphate buffer (0.5 M, pH 7.5) and lysed by take up and expelling the suspension for several (~ 40) times using 23 gauge needle syringe until homogenous solution appeared. Total protein concentration was measured by Bradford method (Sigma) and 20-μg protein was used for each assay as recommended by Spinazzi et al. (2012). The enzymatic activities were performed and calculated as nanomoles per minute per milligram of protein according to the previously described (Spinazzi et al. 2012) method with minor modification of KCN replaced with 100 mM sodium azide which has an action on respiratory chain very similar to cyanide (Bowler et al. 2006; Chen and Lesnefsky 2006). The specific activity of complexes were calculated by subtracting inhibitor-resistant activity (with inhibitor) from total activity (without inhibitor) (inhibitors—C-I: rotenone, C-II: malonate, C-III: antimycin, C-IV: Na-azide).

Cell Viability

For the assessment of effects of treated molecules on cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Briefly, astrocytes were seeded in 96-well plates and after 24-h treatments; the medium was replaced with 100 μl fresh media containing 0.1 mg/ml MTT. After 3 h, MTT was removed and the crystals of formazan were dissolved in DMSO. Formazan was quantified at 570 nm using a microplate reader ELx800-MS (BioTek).

Table. 1 Species-specific oligonucleotide primers list

Target gene	Primer sequence (5′ → 3′)	Annealing temperature (°C)	Amplicon (bp)	Reference
Ndufv2	FP: GCCAGTTGGGAAGTACCACA RP:CCTTGGGTGTCAGATCCTCG	60	224	Present study
Uqere1	FP:TTGACGTTGGCAGTCGCTAT RP:CTCCCGCAGGATCACATCTC	58	290	
MeCP2	FP:CATGGTAGCTGGGATTTAG RP:GAGCTTTCTGATGTCCTGC	60	203	
GAPDH	FP:AGACAGCCGCATCTTCTTGT RP:CTTGCCGTGGGTAGAGTCAT	60	200	[67]

H2.DCF.DA Staining and Fluorimetric Analysis

ROS generation was measured using H2.DCF.DA dye as previously described (Wang and Joseph 1999). In brief, after MeCP2 siRNA transfection, cells were loaded with 100 μM H2.DCF-DA (Sigma, No. D6883) in DMEM in the dark for 30 min in incubator (at 37 °C, 5% CO_2, and 95% O_2). Further, the H2.DCF-DA was removed and cells were washed with 1× PBS and put in 1× HBSS (without Ca^{+2} and Mg^{+2}). Fluorescence was measured by the fluorescence micro plate reader (Synergy HT) at excitation:emission: 485/20: 528/20 wavelengths and gain 35% within 30 min.

Intracellular Calcium Measurement

Following the 24 h MeCP2 siRNA incubation, the cells were incubated with the calcium-sensitive fluorescent dye, Cal 520AM (4 μM) (Abcam, Cat. No. ab171868) dissolved in DMEM (with 1% antibiotic) in the dark for 2 h in incubator (at 37 °C, 5% CO_2, and 95% O_2); then, cells were washed with and put in 1× HBSS (without Ca^{+2} and Mg^{+2}) followed by $[Ca2^{+}]_i$ measurement by the fluorescence micro plate reader (Synergy HT) at excitation:emission: 485/20: 528/20 wavelengths and gain 35% within 30 min.

Measurement of Mitochondrial Membrane Potential

Followed by 24-h MeCP2 siRNA treatment, 0.5 μM rhodamine 123 (cationic dye) (Sigma, No. R8004) dissolved in DMEM (with 1% antibiotic) was incubated in the dark for 30 min in incubator (at 37 °C, 5% CO_2, and 95% O_2); then, cells were washed with and put in 1X HBSS (without Ca^{+2} and Mg^{+2}) followed by MMP measurement by the fluorescence micro plate reader (Synergy HT) at excitation:emission: 485/20: 528/20 wavelengths and gain 35% within 30 min as in Shulyakova (2016).

Statistical Analysis

Data are presented as mean ± SEM of results from three to four independent experiments. Results were analyzed by one-way ANOVA with post-Bonferroni's test (V6.00; GraphPad Prism Software). Statistical significance was considered for $P < 0.05$ ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$).

Results

Characterization of Astrocytes

The astrocytes, post-shaking for second time (DIV 17 to 28), were preferred for the experiments to ensure the mature phenotype. Astroglial cells in culture were confirmed by immunostaining with astrocyte marker-glial fibrilliary acid protein (GFAP) (Fig.1) and Western blot (data not shown). Astrocytes were also confirmed by immunostaining with microglial marker Iba-1, and the cells were found to be negative for the same (data not shown) whereas microglial cells were positive for Iba-1 (Fig.1). Thus, the purity was observed to be 98–100% with negligible fibroblasts like cells during the experiments.

Quercetin Restores Mitochondrial Respiratory Chain Complex mRNA Levels

Firstly, the MeCP2 knock-down was confirmed to be 50% at mRNA level compared to control. Respiratory electron transport chain complexes Uqcrc1 and Ndufv2 genes were onefold upregulated in MeCP2 deficient astrocytes that suggests negative regulation of these genes by MeCP2. Quercetin has been known to modulate many of the gene expressions by modulating transcription factors nuclear factors 1 and 2 (NRF1 and NRF2) and estrogen-related receptors (ERRα) which are involved in regulating nucleus encoded and also mitochondria-encoded respiratory gene expressions (Scarpulla 2006, 2008; Dinkova-Kostova and Abramov 2015; Granado-Serrano et al. 2012; Miyamoto et al. 2011; Galluzzo et al. 2009; Qiu et al. 2018). This suggests ability of quercetin in modulating epigenetic regulators. In our study, we ascertained effect of quercetin on MeCP2 and MeCP2-regulated MRC genes—Uqcrc1 and Ndufv2 mRNA levels. The data show approximately onefold upregulated MeCP2 mRNA level in 100-μM quercetin pre-incubated astrocytes and normalized Uqcrc1 and Ndufv2 mRNA levels compared to MeCP2 knock-down alone (Fig.2). We also checked the brain-derived neurotrophic factor (BDNF) and glial fibrillary acidic protein (GFAP) mRNA and found slightly decreased levels that were normal in 100 μM quercetin-treated MeCP2 knock-down astrocytes. This data suggest that quercetin may have the potential to regulate MeCP2 transcription directly or indirectly.

Quercetin Does Not Alter the Increased Ndufv2 (Complex-I) Protein Expression in MeCP2 Knock-Down Astrocyte

To understand if protein levels are correlated with the mRNA levels, MeCP2 and Ndufv2 protein expressions were determined by Western blot analysis. We observed significant ($P < 0.0001$) reduction in MeCP2 protein level in MeCP2 knock-down astrocytes compared to control. MeCP2 expression remained unchanged in quercetin-treated cells when compared to MeCP2 knock-down alone. Ndufv2 protein level showed subtle upregulation in MeCP2 knock-down astrocytes, whereas it did not alter substantially in quercetin-treated MeCP2 knock-down astrocytes. Thus, MeCP2

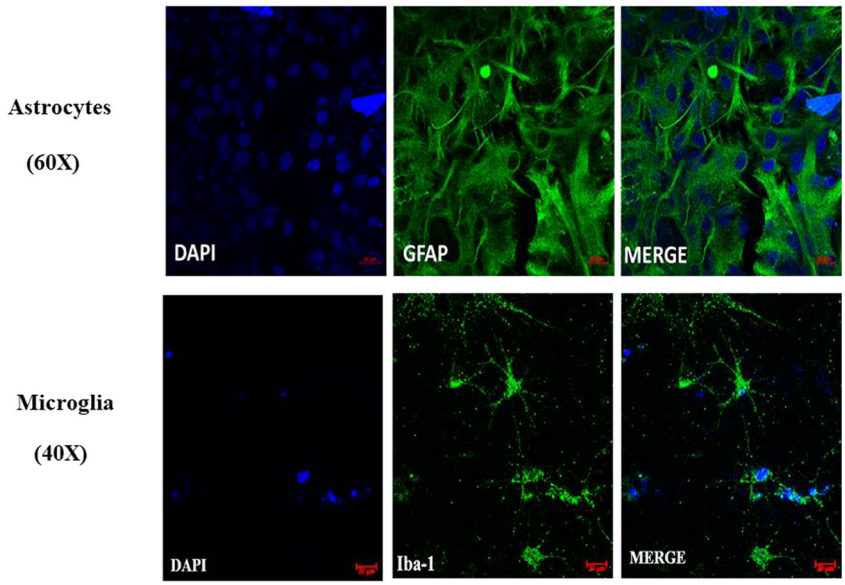

Fig. 1 Characterization of astrocytes. Astrocytes were immunostained with GFAP and microglia were immunostained with Iba-1 primary antibodies followed by FITC-conjugated secondary antibodies. Nucleus was counterstained with DAPI, and cells were imaged under confocal microscope (Carl Zeiss). (Scale bar, 20 μm)

deficiency in astrocytes does not cause difference in mitochondrial respiratory complex protein expression (complex-I-Ndufv2) in higher magnitude that it can be detrimental to cell functioning. Quercetin also does not modulate MRC protein expression (Fig.3).

Quercetin Significantly Increases Mitochondrial Respiratory Complex-II, Complex-III, and Complex-IV Enzymatic Activities

To uncover the contribution of MeCP2 in mitochondrial electron transport chain complex activities, and possible implications of quercetin aglycone on mitochondrial functioning; the spectrophotometric assays for complex-I, complex-II, complex-III, and complex-IV were carried out. Complex-I(NADH:ubiquinone oxidoreductase) enzyme activity was reduced in MeCP2 knock-down astrocytes, that was restored by quercetin (25 μM) in MeCP2 knock-down cells. We also observed decreased enzyme activities of complex-II, complex-III, and complex-IV in MeCP2 knock-down astrocytes. Interestingly, quercetin (25 μM) increased enzyme activities of complex-II, complex-III, and complex-IV also, whereas quercetin (100-μM dose) increased complex-II and complex-III enzyme activities. On the whole, quercetin showed remarkable increase in mitochondrial respiratory complex enzyme activities in MeCP2 knock-down astrocytes while compared to untreated MeCP2 knock-down (Fig.4). Studies in MeCP2-308 mice showed complex-II and complex-V activities significantly reduced when complex-I, complex-II, and complex-V enzyme activities were lowered considerably in MeCP2-bird mouse RTT model (Valenti et al. 2017) that was directly correlated with the ATP levels in brain (De Filippis et al. 2015). In the present study, ATP level was not evaluated but the previous reports in RTT mouse models indicate that increased complex-I and complex-II enzyme activities are interrelated with increase in ATP levels (Valenti et al. 2017). Thus, increased MRC complex activities in quercetin-treated MeCP2 knock-down astrocytes could be extrapolated to be associated with ATP levels.

ROS Production Is Unaltered in MeCP2-Deficient Astrocytes

Surprisingly, unlike the other studies in Rett models and our own observations in C6 glioma cells (data not shown), ROS production was not increased in MeCP2-deficient astrocytes. As astrocytes are central to CNS metabolism, the cell defensive system to ROS production could be more efficient that changed MRC activities that did not increase ROS level (Fig.5).

Quercetin Restores Increased Intracellular Calcium in MeCP2 Knock-Down Astrocytes

Intracellular calcium plays an important role in regulation of calcium-dependent signaling pathways and in cell-cell communication (Clapham 2007; Verkhratsky et al. 2012) Mitochondrial calcium channels also regulate ATP synthesis (Griffiths and Rutter 2009; Brookes et al. 2004; Nicholls 2005). Intracellular calcium was significantly increased in MeCP2 knock-down cells compared to control. Whereas, quercetin (25 and 100 μM) down-regulated intracellular calcium level to the normal level in MeCP2 knock-down cells (Fig.6).

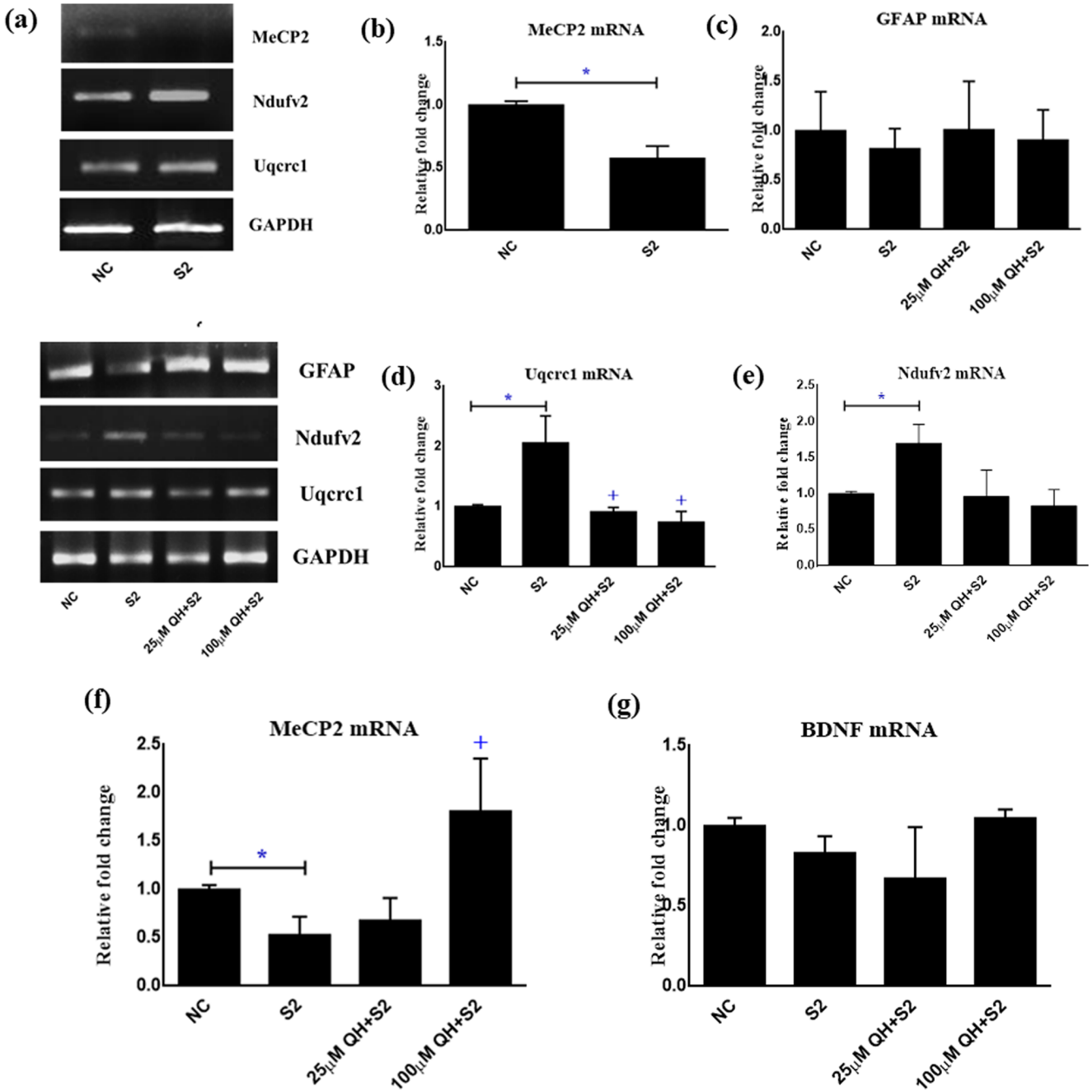

Fig. 2 Effect of quercetin on mitochondrial respiratory chain gene expressions in MeCP2 knock-down astrocytes. Quantitative and semi-quantitative RT-PCR was performed to analyze relative mRNA levels in quercetin-treated and quercetin-untreated MeCP2 siRNA–transfected astrocytes. **a** Representative image of band intensities. **a**–**g** Graphs for MeCP2, GFAP, Uqcrc1, Ndufv2, and BDNF gene expression normalized to GAPDH and compared with control. Data are presented as mean ± SEM ($n = 4$). P value indicated with asterisks ($^*P < 0.05$) is in comparison to negative control (NC) and with plus sign ($+P < 0.05$) is in comparison to MeCP2 knock-down alone (S2)

Quercetin Normalizes the Reduced Mitochondrial Membrane Potential in MeCP2 Knock-Down Astrocytes

A significant loss of mitochondrial membrane potential ($\Delta\Psi$m) renders cells with reduced ATP level and subsequent death. During cellular stress, $\Delta\Psi$m may be altered by dysregulation of intracellular ionic flux (e.g., Ca^{+2}, K^{+2}) (Nicholls 2006). Mitochondrial dysfunction has been implicated in the pathophysiology of many diseases; therefore, the ability to determine $\Delta\Psi$m can provide important clues about the status of the cell and the function of mitochondria (Perry et al. 2011). Moreover, quercetin-reversing effect on mitochondrial membrane potential in disease conditions has been documented (Qiu et al. 2018; Carrasco-Pozo et al. 2012).

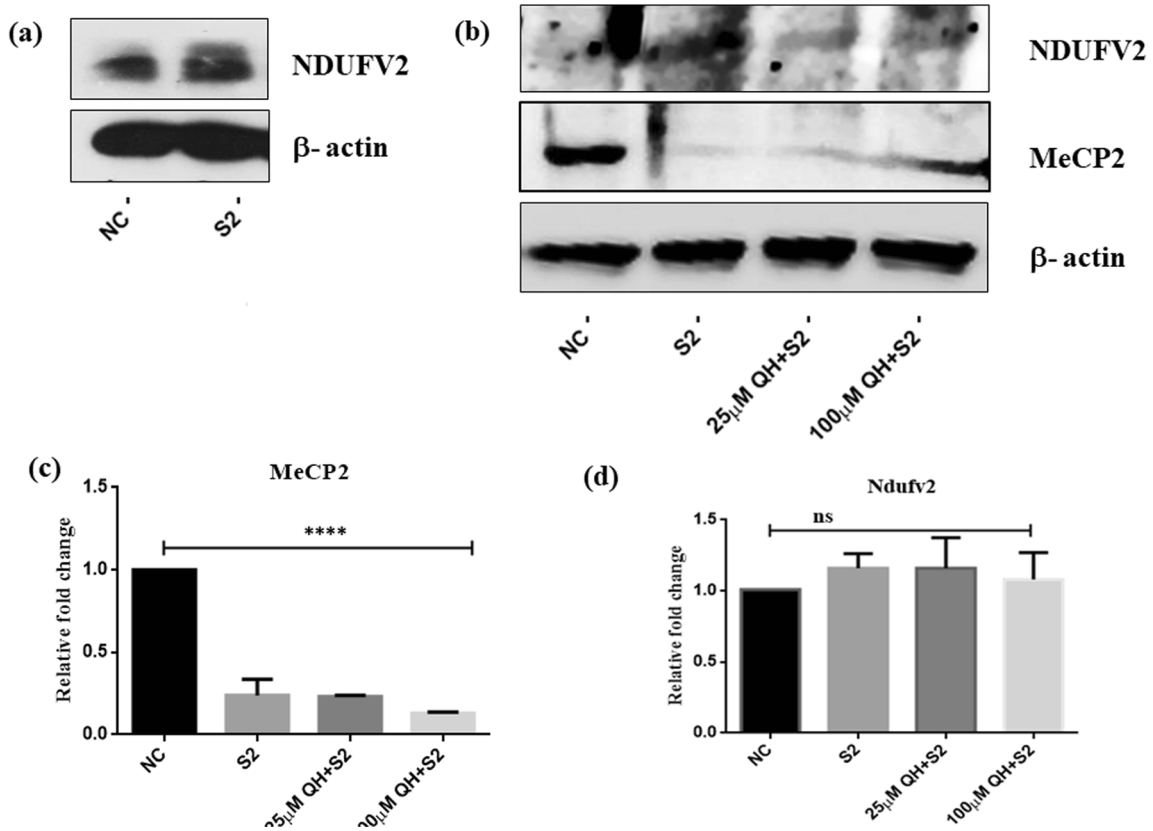

Fig. 3 Effect of quercetin on mitochondrial respiratory chain protein expression in MeCP2 knock-down astrocytes. Western blot analysis was performed to analyze relative protein levels in quercetin-treated and quercetin-untreated MeCP2 siRNA–transfected astrocytes. **a**, **b** Representative image of band intensities of MeCP2, Ndufv2, and β-actin as internal control. **c** Graph for MeCP2. **d** Graph for Ndufv2 protein expressions normalized to β-actin and compared with control. Data are presented as mean ± SEM ($n = 4$); P value indicated with asterisks (****$P < 0.0001$) and ns (non-significant) are in comparison to negative control (NC).There was no significant change in quercetin-treated groups in protein expressions as compared to MeCP2-deficient astrocytes

In this study, mitochondrial membrane potential was measured by cationic dye rhodamine 123 in non-quenching mode as 0.5-μM concentration was used. Briefly, When the mitochondria is in depolarized state (i.e., interior is less negative), rhodamine123 will accumulate less and read-out will be less fluorescence intensity, whereas in hyperpolarized state (i.e., interior is more negative), higher accumulation of rhodamine123 will occur that show more fluorescence intensity (Perry et al. 2011; Baracca et al. 2003). Thus, under these conditions, the mean fluorescence intensity of MeCP2 knocked-down astrocytes (S2) was reduced ($P < 0.05$) compared to control that suggests depolarized state in MeCP2 knocked-down astrocytes (S2) which means lowered mitochondrial respiratory chain activity. In quercetin (25, 100 μM)-treated MeCP2 knocked-down astrocytes, mitochondrial membrane potential was increased ($P < 0.01$) compared to untreated MeCP2 knocked-down astrocytes (S2) (Fig.7). Thus, quercetin higher micromolar dose indicated the potential to rescue impaired mitochondrial respiratory chain activity. In this study, the possibility of altered mitochondrial morphology and mass (as they can interfere with absorption of dye) were not validated. Also, the pharmacological inhibitors were not used to confirm the results.

Quercetin Treatment and MeCP2 Deficiency Do Not Affect Cell Viability

Neither MeCP2 nor quercetin treatments caused cell death evaluated by MTT assay. This indicated that the mitochondrial dysfunction caused by MeCP2 deficiency was not toxic to cell death. Also, the quercetin doses used were not detrimental with respect to cell viability (Fig.8).

Discussion

Previous reports have shown mitochondrial abnormalities in whole brain, different brain regions, or neurons of RTT murine models or patients, but a very few studies have been done in MeCP2-deficient astrocytes per se. Mitochondrial impairment has been observed in the symptomatic MeCP2$^{y/-}$ condition but not in asymptomatic RTT which indicates

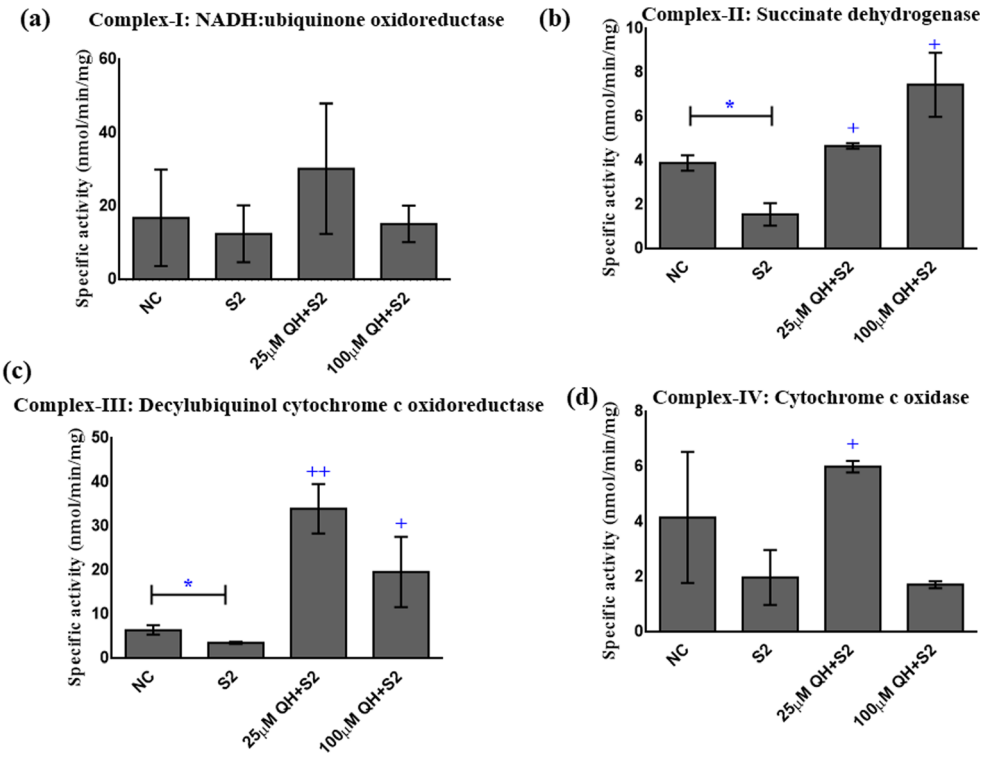

Fig. 4 Effect of quercetin on mitochondrial respiratory chain enzyme activities in MeCP2 knock-down astrocytes. Spectrophotometric analysis revealed significant difference in MeCP2 siRNA–transfected and quercetin-treated MeCP2-transfected astrocytes compared to negative control (NC) and MeCP2 knock-down alone (S2), respectively. **a**–**d** Graph for complex-I, complex-II, complex-III, and complex-IV enzyme activities. Data represent mean-specific activity in nanomoles per minute per milligram of total protein ± SEM ($n = 4$–6). P value indicated with asterisks ($^{*}P < 0.05$) is in comparison to negative control (NC) and with plus sign ($+P < 0.05$, $++P < 0.01$) is in comparison to MeCP2 knock-down alone (S2)

mitochondrial involvement in disease progression (Kriaucionis et al. 2006; Gold et al. 2014; Valenti et al. 2017).

As the ATP is the main energy source for regulation of cell function and regulation of various signaling pathways dependent of phosphorylation for their activation, its lowered production imbalances homeostasis of cell. To what extent mitochondrial impairment plays a role in disease progression

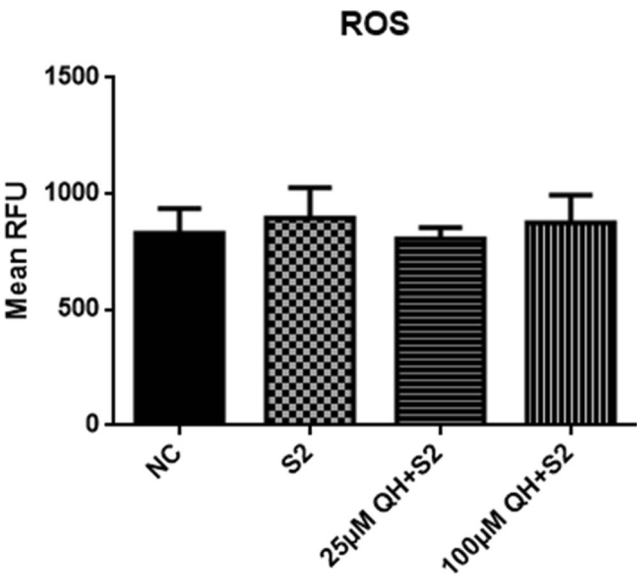

Fig. 5 Effect of quercetin on ROS production in MeCP2 knock-down astrocytes. Cells after 24-h MeCP2 siRNA transfection were incubated for 30 min with H2.DCF.DA fluorescent dye. Graph for ROS production depicts no change in ROS production in MeCP2 knock-down (S2) or quercetin-treated (QH + S2) groups. Data is represented as mean relative fluorescence unit ± SEM (RFU) ($n = 4$)

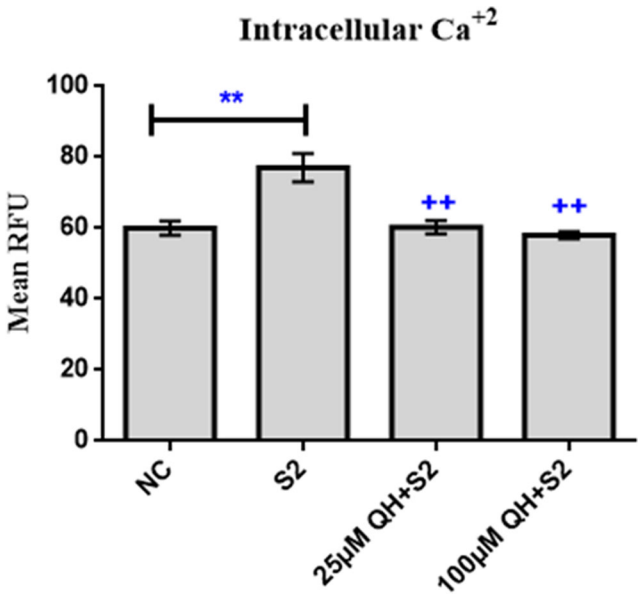

Fig. 6 Effect of quercetin on intracellular calcium in MeCP2 knock-down astrocytes. Intracellular calcium was measured by fluorimetric analysis of calcium-binding fluorescent dye Cal520AM. Graph for intracellular calcium shows significant differences. Data is represented as mean relative fluorescence unit (RFU) ± SEM ($n = 4$). P value indicated with asterisks ($^{**}P < 0.01$) is in comparison to negative control (NC) and with plus sign ($++P < 0.01$) is in comparison to MeCP2 knock-down alone (S2)

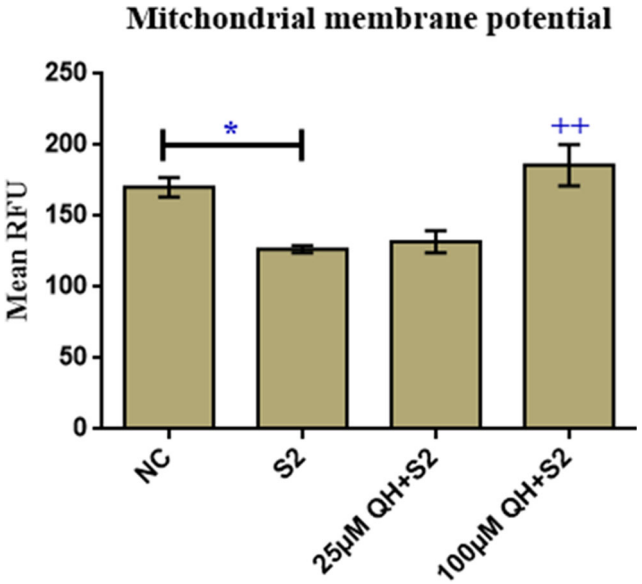

Fig. 7 Effect of quercetin on mitochondrial membrane potential in MeCP2 knock-down astrocytes. Mitochondrial membrane potential was measured by fluorimetric analysis of cationic dye rhodamine123 in non-quenching mode. Graph shows significant differences. Data is represented as mean relative fluorescence unit (RFU) ± SEM ($n = 4$). P value indicated with asterisks ($^*P < 0.05$) is in comparison to negative control (NC) and with plus sign ($^{++}P < 0.01$) is in comparison to MeCP2 knock-down alone (S2)

or phenotypic expression in Rett syndrome is still in gray area. Also, whether the mitochondrial impairment is solely by direct involvement of MeCP2 in mitochondrial gene transcription or indirect effects due to dysregulated phosphorylation of

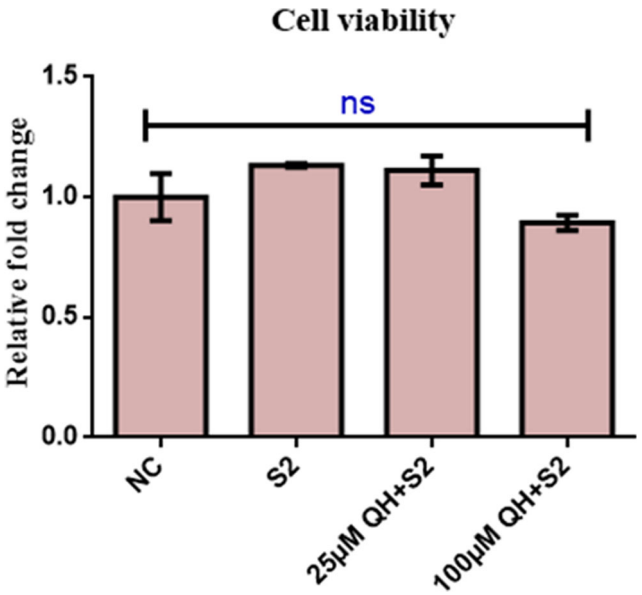

Fig. 8 Cell viability analysis of quercetin-treated and MeCP2 knock-down astrocytes. Cell viability was assessed by MTT assay. Graph shows no fold change in viability relative to control (NC). Data represented as mean ± SEM ($n = 4$)

signaling pathways owing to impaired ATP synthesis is also largely unclear. Astrocytes have the lowest MeCP2 expression among all the brain cells which could be due to the multifaceted roles of astrocytes that are fulfilled by higher gene expressions (at a given time).

Previous reports have shown ≥ 1.5-fold altered transcripts of MRC genes in microarray analysis of RETT patients (Pecorelli et al. 2013) and in ADDER differential display and real-time PCR analysis in MeCP2$^{-/y}$ mouse model which is directly correlated with the disease status (Kriaucionis et al. 2006). Only a single study has shown direct binding of MeCP2 to Uqcrc1 promoter (Kriaucionis et al. 2006), and the information regarding direct or indirect modulatory effects of MeCP2 on ETC genes is not completely available. In addition to transcription factors NRFs and ERRs, stimulatory protein 1(Sp1) and yin yang 1 (YY1) have also been linked to many genes required for mitochondrial respiratory chain complex gene expression and functions (Scarpulla 2008). PGC-1α, a transcription factor regulating the expression of antioxidants such as SOD1 and mitochondrial biogenesis, was also found to be reduced in symptomatic MeCP2$^{-/y}$ mice (Gold et al. 2014). In other study, NRF-1, Tfam, PGC-1α, and CREB transcription factors involved in mitochondrial respiratory complexes were found to be severely modulated at protein level in RTT patient cells (Cervellati et al. 2015). MeCP2 interaction with co-activators or co-repressors involved in mitochondrial respiratory chain genes could be responsible for the modulation. Our data show effect of quercetin on Uqcrc1 and Ndufv2 genes in MeCP2 knock-down that could be through regulation of related transcription factors.

Studies in MeCP2$^{-/y}$ bird mouse model (Kriaucionis et al. 2006) and MeCP2-308 mouse model (Valenti et al. 2017) also showed insignificant alteration in mitochondrial respiratory chain complex protein expression, whereas complex-I and complex-II proteins were found to be reduced in the striatum of the brain in MeCP2-308 mice (De Filippis et al. 2015).

In current study, in MeCP2-deficient astrocytes, all the complex activities were found to be reduced; whereas in RTT models, not all the complex activities were affected. Kriaucionis et al. (2006) reported indistinguishable complex-I and decreased complex-IV activity in in gel assays and increased respiratory rates in polarographic oxygen electrode study and concluded that the faster consumption of oxygen could be due to the reduced respiratory efficiency. Reduction in cytochrome oxidase subunit 1 (MTCO1) at transcript and protein levels and significantly reduced complex-II + III and complex-IV enzyme activities were observed in the skeletal muscle tissue isolated from symptomatic MeCP2^{tm1Tam} mouse model (Gold et al. 2014). Reduced enzymatic activities of MRC complexes were also observed in the cerebellum of the MeCP2$^{y/-}$ mice (Gold et al. 2014). Microarray analysis revealed twofold reduced cardiolipin synthase 1 (Crls1) expression fold in the skeletal muscle of the symptomatic

MeCP2$^{y/-}$ mice compared to their wild-type litter mates. Crls1 plays a role in mitochondrial phospholipid cardiolipin biosynthesis; cardiolipin interacts with respiratory chain complexes and stabilizes their organization in inner membrane of the mitochondria. Thus, there could be multiple targets for deterioration of mitochondrial respiratory chain activities (Gold et al. 2014).

MRC functionality is associated with ROS production, and in number of reports, ROS overproduction or altered ROS regulatory enzymes/genes have been documented in RTT models (Valenti et al. 2017; Großer et al. 2012; De Filippis et al. 2015; De Felice et al. 2014) and patients (Pecorelli et al. 2013; Pecorelli et al. 2016; Cervellati et al. 2015; Signorini et al. 2014; De Felice et al. 2012; Ciccoli et al. 2012; Leoncini et al. 2011; De Felice et al. 2009; Sierra et al. 2001). However, normalizing MRC activities has been found to be significantly preventing ROS overproduction in RTT mouse model (Valenti et al. 2017). Besides, mitochondrial membrane potential was also observed to be depolarized in RTT mouse models in previous reports (Großer et al. 2012; De Filippis et al. 2015).

Quercetin, a well-known antioxidant has been explored in many of the diseases involving the mitochondria, but ours is the first report exploring its effects in mitochondrial dysfunction caused by MeCP2 deficiency in astrocytes. In current study, data show increased Uqcrc1 and Ndufv2 gene expression with minor upregulated Ndufv2 protein expression in MeCP2 knock-down astrocytes. In spite of the upregulated gene expression, the enzyme activities were significantly reduced in MeCP2 knock-down astrocytes that suggest that other pathways might be operative in regulating MRC enzyme activities, and hence, gene expressions are not directly correlated with the enzyme activities. The increased intracellular calcium and decreased mitochondrial membrane potential are concomitant with the decreased MRC enzyme activities. Quercetin also normalized MRC gene expression but increased MRC enzyme activities compared to MeCP2 knock-down alone that indicate quercetin's multiple targets to ameliorate the imbalance in context-dependent manner.

Further studies are needed in in vivo to assess protective effects of quercetin on phenotype or progression of symptoms in RTT models and to understand the translational relevance in diseases associated with MeCP2 mutation or deficiency.

As a whole, the results confirm mitochondrial dysfunction in MeCP2-deficient astrocytes and positive implication of quercetin in restoring the function. As there is no direct therapy available for MeCP2 deficiency–involved diseases up to date, improving the downstream parameters might manifest the slowed progression or symptoms of the diseases. Finally, the data from the current study provides some clue about the pathogenesis of Rett syndrome in regard to mitochondrial dysfunction in glial cells and the importance of therapeutic applications of quercetin.

Acknowledgements We acknowledge the animal house facility, Biochemistry department, Faculty of Science, The Maharaja Sayajirao University of Baroda and Sun Pharma, Baroda for the animals. We are also thankful to DBT-ILSPARE program for the confocal microscope and real-time PCR facility in the Dr. Vikram Sarabhai block. We would also like to thank Dr. R.V. Devkar for technical support.

Funding Information We thank Gujarat State Biotechnology Mission (GSBTM), Gandhinagar, India, for funding this research and providing JRF-SRF during May 2013–2016. (Sanction order No. GSBTM/MD/PROJECTS/SSA/3379/12-13dated 4th March 2013).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Animal Ethical Statement All the mentioned studies were approved by institutional animal ethics committee (IAEC), Department of Zoology & Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda.

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