Chapter-III

To determine the effects of quercetin on mitochondrial dysfunction mediated by MeCP2 deficiency in astrocytes

3.1 Introduction

The electron transport chain (ETC) is coded by more than 850 nuclear DNA (nDNA) genes and 37 mitochondrial DNA (mtDNA) genes (Chauhan, Gu, and Chauhan 2012). These genes are regulated by number of nuclear encoded transcription factors such as NRF1, NRF2, ERR, Tfam etc. and co-activators like PGC-1 family proteins (Scarpulla 2006, 2008, Friedman and Nunnari 2014). Methyl-CpG-binding protein 2 (MECP2) is a global transcription factor, known to regulate wide array of genes positively and negatively depending on the genome context in which it is operated (Chahrour et al. 2008, Ben-Shachar et al. 2009). Thus, it is more likely that many of the electron transport chain genes are regulated by MeCP2.

Several reports provide an insight into mitochondrial dysfunction in brain of MeCP2 null mouse and RTT syndrome patients reviewed in detail by (Shulyakova et al. 2017). Altered mitochondrial respiratory chain complexes 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) in RTT indicates potential role of mitochondrial impairment in RTT pathogenesis. Also, the altered ROS stabilizing enzymes such as superoxide dismutase, catalase, Glutathione S-transferase etc. have been observed in RTT condition (Gold et al. 2014, Großer et al. 2012, Pecorelli et al. 2013) that supports the notion of mitochondrial involvement in disease.

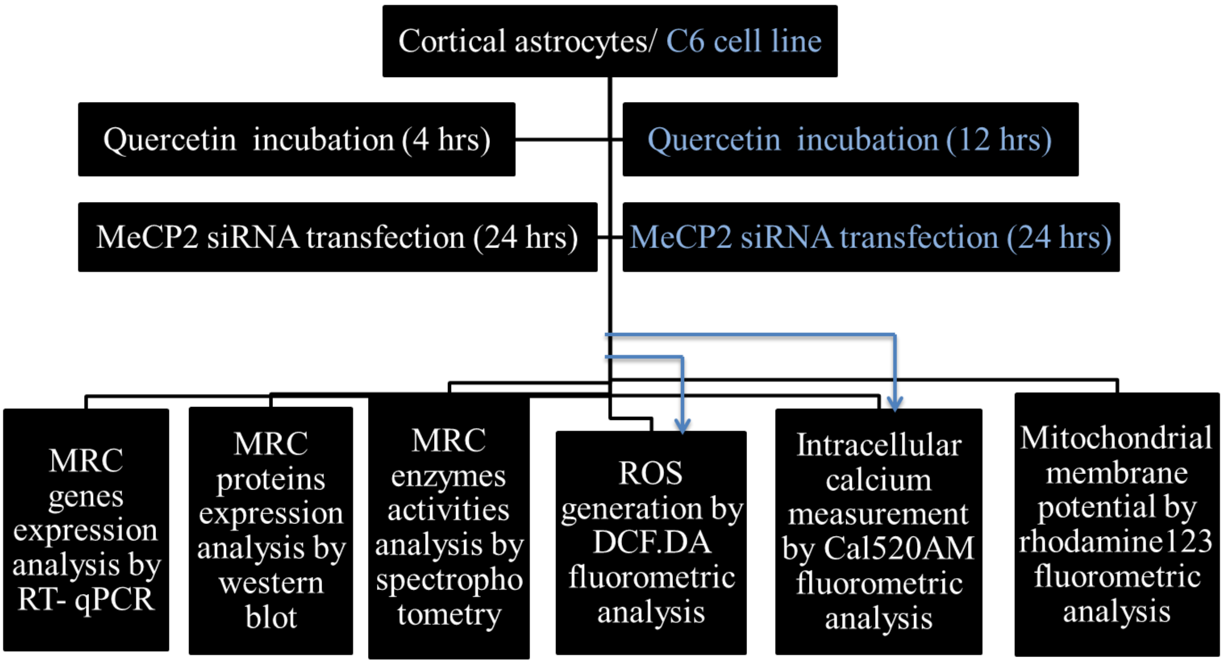
Electron transport chain is also responsible for buffering the signal ion Ca^{2+} (Friedman and Nunnari 2014) and mitochondrial dysfunction is also attributed to impaired calcium flux (Nicholls 2005). There are growing body of evidences that suggest role of MeCP2 in calcium homeostasis in neurons (Mironov et al. 2009, Marchetto et al. 2010). Cytosolic calcium was found to be higher in resting state preBötC respiratory neurons in $MeCP2^{-/y}$ mouse model of Rett syndrome, which was improved by application of BDNF, a growth factor under the control of MeCP2. Also, the bigger amplitudes and longer decay time of depolarization- induced calcium transients were observed in MeCP2 knockout mouse model (Mironov et al. 2009) and the frequency of intracellular calcium transients was decreased in RTT neurons (Marchetto et al. 2010). Moreover, calcium dependent kinase CaMKII phosphorylates MeCP2 (S421 site) in isolated neurons from wild type or MeCP2 knockout mice (Buchthal et al. 2012). Thus, there is an interconnecting link between MeCP2 activation and calcium signalling. In addition, Rett syndrome astrocytes were found to be spreading MeCP2 deficiency through gap junctions (Maezawa et al. 2009) that points to the involvement of astrocytic calcium waves in this communication.

Quercetin, a plant favonol, is a widely studied compound having neuroprotective role (Costa et al. 2016) and modulatory effects on intracellular signalling pathways and epigenetic mechanisms *in vitro* and *in vivo* (Cacabelos and Torrellas 2015, Williams, Spencer, and Rice-Evans 2004). Quercetin is effective in improving mitochondrial dysfunctions in many of the diseases (Qiu, Luo, and Chen 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, Ca^{2+} ATPase, that transfers Ca^{2+} from the cytosol of the cell to the lumen in MeCP2 deficient neurons (Mironov et al. 2009). Interestingly, quercetin has been documented to up regulate (BDNF) in Alzheimer mouse model (Hou et al. 2010) and in acute hypobaric hypoxia (HH) condition (Liu et al. 2015). Hence, it was hyposthesized that increased BDNF expression following quercetin treatment in MeCP2 deficient astrocytes benefits in impaired calcium homeostasis.

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, Tripathi, and Pillai 2017, Sharma et al. 2015, Vora et al. 2010, Yasui et al. 2013, Nguyen et al. 2013, Maezawa et al. 2009). Although the MeCP2 expression in glial cells is low in general and 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 genes expression in oligodendrocytes-dorsal root ganglion (DRG) neurons- astrocytes co-culture (Lipi, Jaldeep, and Prakash 2018).

As astrocytes support neurons by regulating metabolic functions (Wang and Bordey 2008), the present study aimed to understand whether mitochondrial respiratory chain complexes genes, protein and enzyme activities are altered in MeCP2 deficient astrocytes and whether these can be further ameliorated by quercetin treatment. To this aim the MeCP2 knock-down in astrocytes by siRNA transfection was performed and effect of pre-treated quercetin (25μM, 100 μM) in the same was observed. Also, whether Ca^{2+} homeostasis, ROS generation and mitochondrial membrane potential too are affected as a consequence of impaired mitochondrial respiratory chain(MRC) impairment and if quercetin can improve these were checked. The investigation on the expression of genes encoding mitochondrial respiratory complex subunits i.e. Ubiquinol cytochrome c reductase core protein (Uqcrc1) and NADH dehydrogenase (ubiquinone) flavoprotein 2 (Ndufv2), protein (Ndufv2) and mitochondria electron transport chain complexes' enzymes activities were carried out by RT-PCR, western blot and spectrophotometric assays respectively in quercetin treated MeCP2 knock-down astrocytes. Intracellular Ca^{2+} level, ROS and mitochondrial membrane potential were determined by fluorometric measurement of Cal520AM, DCF.DA and Rhodamine123 respectively in quercetin treated MeCP2 deficient astrocytes as well as C6 glial cells.

3.2 Experimental design



3.3 Results and discussion

3.3.1 Characterization of cortical astrocytes and C6 cell line

In *in vivo* condition, most astrocyte proliferation largely completes by P14 (Burns et al. 2009). In *in vitro*, the mix glial culture proliferate maximum until day *in vitro*-10 (DIV 10) then they start to mature (Schildge et al. 2013). Hence, post-shaking for second time (DIV 17 to 28), the astrocytes were preferred for the experiments to ensure the mature phenotype of isolated astrocytes. Primary astrocytes isolated and cultured from rat brain cortex reflect many astrocyte characteristics, makes it an ideal model for the study (Schildge et al. 2013). Astrocytes are identified by intermediate filaments (glial fibrils) and glial fibrillary acidic protein (GFAP), being the major component of glial fibrils, is exclusive for astrocytes in the CNS (Skoff and Knapp 1991). Thus, isolated cortical astrocytes were characterized by immunocytochemistry, RT-PCR and western blotting analysis of GFAP marker (Fig.3.1, Fig.3.2 & Fig.5.3). The rat C6 glial cell line was cloned from a chemically induced rat brain tumour and was classified as an undifferentiated astrocytic cell type (Benda et al. 1968). The C6 glial cell line has been employed as an *in vitro* model for the study of glial cell behaviour (Trentin and Alvarez-Silva 1998). Thus, C6 glial cells were also characterized by immunocytochemistry and RT-PCR analysis of GFAP, an astrocytic marker protein (Fig.3.1). Both the cell types were found to be GFAP positive which confirms astrocytic lineage of these cells.

3.3.2 Purity of astrocytes

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.3.2). Thus, the purity was observed to be 98-100% with negligible fibroblasts like cells during the experiments.

3.3.3 Quercetin treatment and MeCP2 deficiency do not affect cell viability

Neither MeCP2 deficiency nor quercetin treatments caused cell death as observed in 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. 3.3).

3.3.4 Effect of quercetin on morphology of astrocytes

Astrocytes morphology did not change in either MeCP2 knock-down or 25μM quercetin treated cells. Interestingly, the cells were found with slender processes in 100 μM quercetin treated cells (Fig. 3.3.b).

3.3.5 Quercetin restores mitochondrial respiratory chain complex-III (Uqcrc1) mRNA level

Firstly, the MeCP2 knock-down was confirmed to be 50% at mRNA level compared to negative control. Respiratory electron transport chain complexes Uqcrc1 and Ndufv2 genes were one fold up-regulated in MeCP2 deficient astrocytes that suggests negative regulation of these genes by MeCP2. Quercetin has been known to modulate many of the genes expression by modulating transcription factors nuclear factors 1 & 2 (NRF1 and NRF2) and estrogen related receptors (ERRα) which are involved in regulating nucleus encoded and also mitochondria encoded respiratory genes expression (Scarpulla 2006, 2008, Dinkova-Kostova and Abramov 2015, Granado-Serrano et al. 2012, Miyamoto et al. 2011, Galluzzo et al. 2009, Qiu, Luo, and Chen 2018). These observations suggest ability of quercetin in modulating epigenetic regulators. In this study, the effect of quercetin on MeCP2 and MeCP2 regulated MRC genes- Uqcrc1 and Ndufv2 mRNA levels were ascertained. The data show approximately 1-fold up-regulated MeCP2 mRNA level in 100μM quercetin pre-incubated astrocytes and normalised Uqcrc1 and Ndufv2 mRNA levels compared to MeCP2 knock-down alone (Fig.3.4). Brain derived neurotrophic factor

(BDNF) and Glial fibrillary acidic protein (GFAP) mRNA were also checked and found slightly decreased levels in MeCP2 knock-down astrocytes which 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.

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 has not been investigated extensively. 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 complexes genes 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 patients 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. Thus, data show effects of quercetin on Uqcrc1 and Ndufv2 genes in MeCP2 knock-down astrocytes, that could be through regulation of related transcription factors.

3.3.6 Quercertin does not alter the increased Ndufv2 (complex-I) protein expression in MeCP2 knock down astrocyte

To understand if protein levels are co-related with the mRNA levels, MeCP2 and Ndufv2 proteins expressions were determined by western blot analysis. Significant ($p<0.0001$) reduction in MeCP2 protein level in MeCP2 knock-down astrocytes compared to control was observed. MeCP2 expression remained unchanged in quercetin treated cells when compared to MeCP2 knock down alone. Ndufv2 protein level showed subtle up-regulation in MeCP2 knockdown astrocytes whereas; it was not altered substantially in quercetin treated MeCP2 knock-down astrocytes. Thus, MeCP2 deficiency doesn't cause Ndufv2 higher magnitude increase in astrocytes. Quercetin treatement also does not have any effect on Ndufv2 protein expression irrespective of alteration in mRNA level (Fig.3.5).

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

3.3.7 Quercetin significantly increases mitochondrial respiratory complexes- II, III and IV enzymatic activities

To analyse the contribution of MeCP2 in mitochondrial electron transport chain complexes activities, and possible implications of quercetin on mitochondrial functioning, the spectrophotometric assays for complex-I, II, III and 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. Decreased enzyme activities of complex-II, III, and IV in MeCP2 knock-down astrocytes were observed. Interestingly, quercetin (25 μM) increased enzyme activities of complex-II, III, and IV also whereas; quercetin (100 μM dose) increased complex- II and III enzyme activities. On the whole, quercetin showed remarkable increase in mitochondrial respiratory complexes enzyme activities in MeCP2 knock-down astrocytes compared to untreated MeCP2 knock-down astrocytes (Fig.3.6). Studies in MeCP2-308 mice and MeCP2-bird mouse RTT model showed significantly reduced complex-II and V activities and complex- I, II and V enzyme activities respectively (Valenti et al. 2017). These activities changes were 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 & II enzyme activities are interrelated with increase in ATP levels (Valenti et al. 2017). Thus, increased MRC complexes activities in quercetin treated MeCP2 knock-down astrocytes could be extrapolated to be associated with ATP levels.

3.3.8 ROS production was unaltered in MeCP2 deficient astrocytes

Surprisingly, unlike the other studies in Rett models and our laboratory observations in C6 glioma cells (Fig.3.10), 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 did not increase ROS level (Fig.3.7).

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).

3.3.9 Quercetin restores increased intracellular calcium in MeCP2 knock-down astrocytes

Intracellular calcium plays an important role in regulation of calcium dependent signalling pathways and in cell-cell communication (Clapham 2007, Verkhratsky, Rodríguez, and Parpura 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 & 100μM) down-regulated intracellular calcium level to the normal control in MeCP2 knock-down cells (Fig.3.8).

Increase in cytosolic calcium may be due to Ca^{2+} entry through plasma membrane(from the extracellular matrix) or Ca^{2+} release from intracellular stores or both.The cytosolic Ca^{2+} level normalizes by the ATP driven pumps or antiporters. Not only endoplasmic reticulum (ER) and mitochondria but also golgi apparatus, lysosomes, secretary vesicles, peroxisomes and endosomes play a role in Ca^{2+} signalling (Contreras et al. 2010). Moreover, cytosolic Ca^{2+} can affect the activity of mitochondrial enzymes such as glycerophosphate dehydrogenase, or the malate aspartate shuttle and glutamate/malate dependent respiration through activation of the aspartate-glutamate carriers.However, studies demonstrate that increase in cytosolic Ca^{2+} is accompanied by increased Ca^{2+} level in mitochondria. This excess Ca^{2+} uptake by mitochondria causes bioenergetics failure and thus impaired mitochondrial functions (Contreras et al. 2010). Thus, increased intracellular calcium is related to mitochondrial respiratory chain complexes dysfunction in untreated and quercetin treated MeCP2 knock-down cells.

3.3.10 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 ΔΨm can provide important clues about the status of the cell and the function of mitochondria (Perry et al. 2011). 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). Moreover, quercetin reversing on mitochondrial membrane potential in disease conditions has been documented (Qiu, Luo, and Chen 2018, Carrasco-Pozo et al. 2012).

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 fluroscence intensity of MeCP2 knock-down astrocytes (S2) was reduced ($p<0.05$) compared to control that suggests depolarized state in MeCP2 knock-down astrocytes (S2) which means lowered mitochondrial respiratory chain activity. In quercetin (25, 100μM) treated MeCP2 knock- down astrocytes, mitochondrial membrane potential was increased ($p<0.01$) compared to untreated MeCP2 knock-down astrocytes (S2) (Fig.3.9). Thus, quercetin higher micromolar dose demonstrated the potential to rescue impaired mitochondrial respiratory chain activity.

3.3.11 Quercetin alters intracellular calcium, ROS but not cell viability in MeCP2 knock down C6 glial cell line

Intracellular calcium, ROS generation and apoptosis are inter-related because of involvement of mitochondria in all the three processes (Ermak and Davies 2002, Zhivotovsky and Orrenius 2011, Görlach et al. 2015). In present study, intracellular calcium was found to be significantly higher in MeCP2 knock-down cells in comparison to control. Various QH concentrations (25, 50, 100, 200 μM) were applied to control and MeCP2 siRNA treated cells both to check QH's context-dependent effect. Intriguingly, QH (25,50,100 μM) showed no effect on $[Ca^{2+}]_i$ in the control group but it lowered $[Ca^{2+}]_i$ in MeCP2 knock-down cells compared to untreated MeCP2 knock-down cells. Thus, QH was found to modulate the cytosolic calcium level to match environmental challenge (MeCP2 deficiency). However, QH (200 μM) elevated $[Ca^{2+}]_i$ in control and MeCP2 knock-down cells that exhibit hormesis phenomenon. ROS was observed to be substantially increased in MeCP2 knock-down cells in comparison to control. Besides, QH (25,50,100 μM) acted as pro-oxidant in MeCP2 knock-down cells while compared to untreated MeCP2 knock-down cells but QH (200 μM) dropped ROS to baseline (control) in MeCP2 knock-down cells.

Apart from that the cell viability was also assessed and no difference was observed in any of the groups (Fig.3.10).

Mitochondrial electron transport chain genes being regulated by MeCP2 and deregulated in MeCP2 deficient condition (Kriaucionis et al. 2006, Pecorelli et al. 2013, Gold et al. 2014) may also lead to impaired ROS generation and reduced ATP production (Valenti et al. 2017, De Filippis et al. 2015, Bebensee, Can, and Müller 2017, Jin et al. 2015). Additionally, oxidative stress has been reported to increase resting $[Ca^{2+}]_i$ by disrupting Ca^{2+} receptor (Tang et al. 2013). Consequences of disturbed calcium homeostasis are likely to be many; one of them could be deregulation of ATP production. The data in C6 glial cells showed that QH down regulated the $[Ca^{2+}]_i$ but did not reduce ROS and display no effect on cell viability. This suggests that QH might have pleiotropic effects in MeCP2 deficient glial cells.

Fig. 3.1 Characterization of astrocytes and C6 glial cells (a) Cells were immunostained with anti-GFAP primary antibody followed by FITC-conjugated secondary antibody. Nucleus was counterstained with DAPI and cells were imaged under confocal microscope (Carl zeiss). (Scale bar: 20μm) (b) Semi-quantitative RT-PCR for GFAP was performed using specific primers. Representative agarose gel images show band intensities for GFAP and GAPDH (internal control) of astrocytes and C6 glial cells.

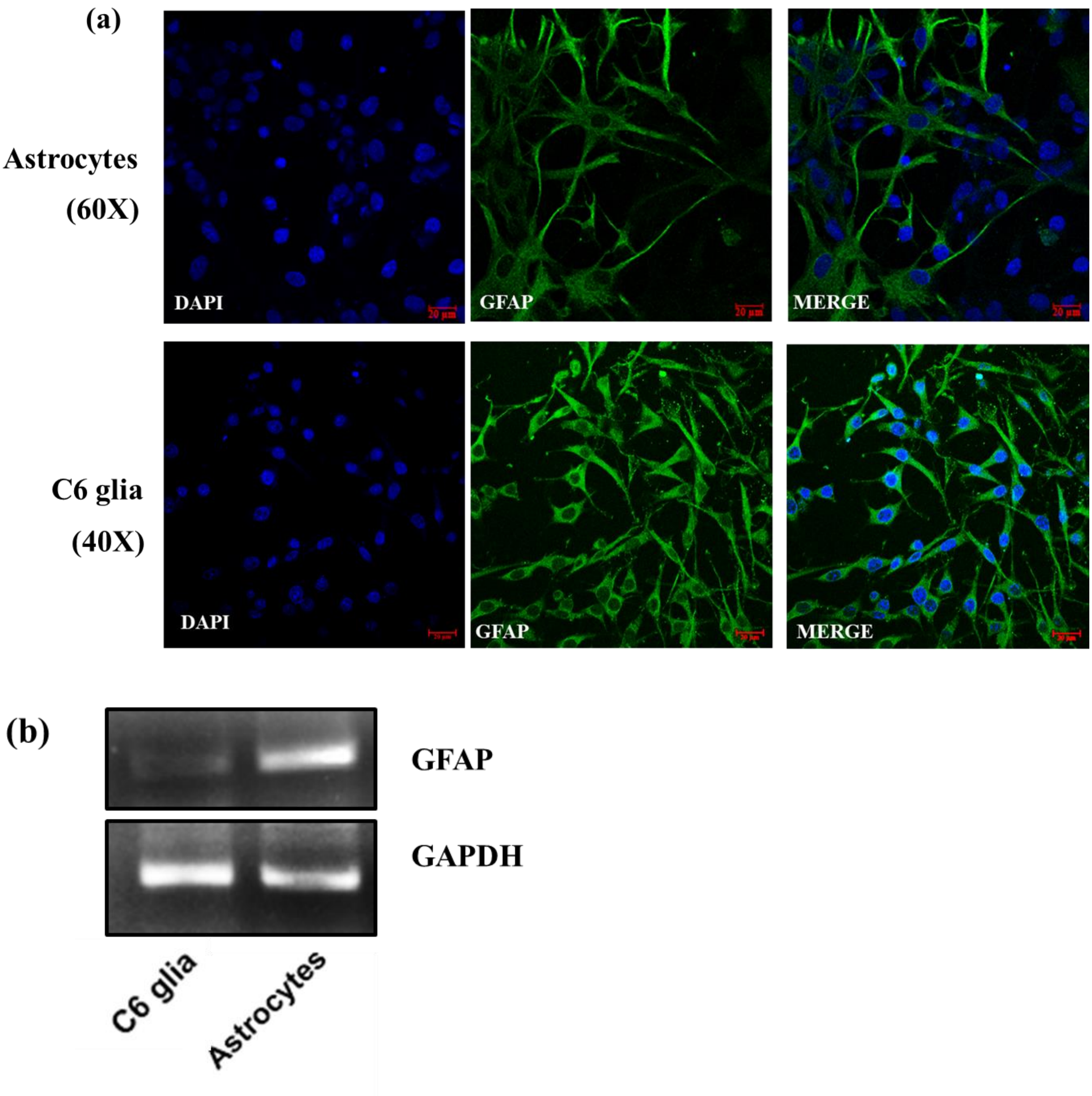


Fig.3.2 Confirmation of purity of astrocytes Astrocytes were immunostained with GFAP and microglia were immunostained with Iba-1 primary antibodies followed by FITC-conjugated secondary antibodies. Nucleus was counterstained with DAPI and cells were imaged under confocal microscope (Carl zeiss). (Scale bar: 20µm)

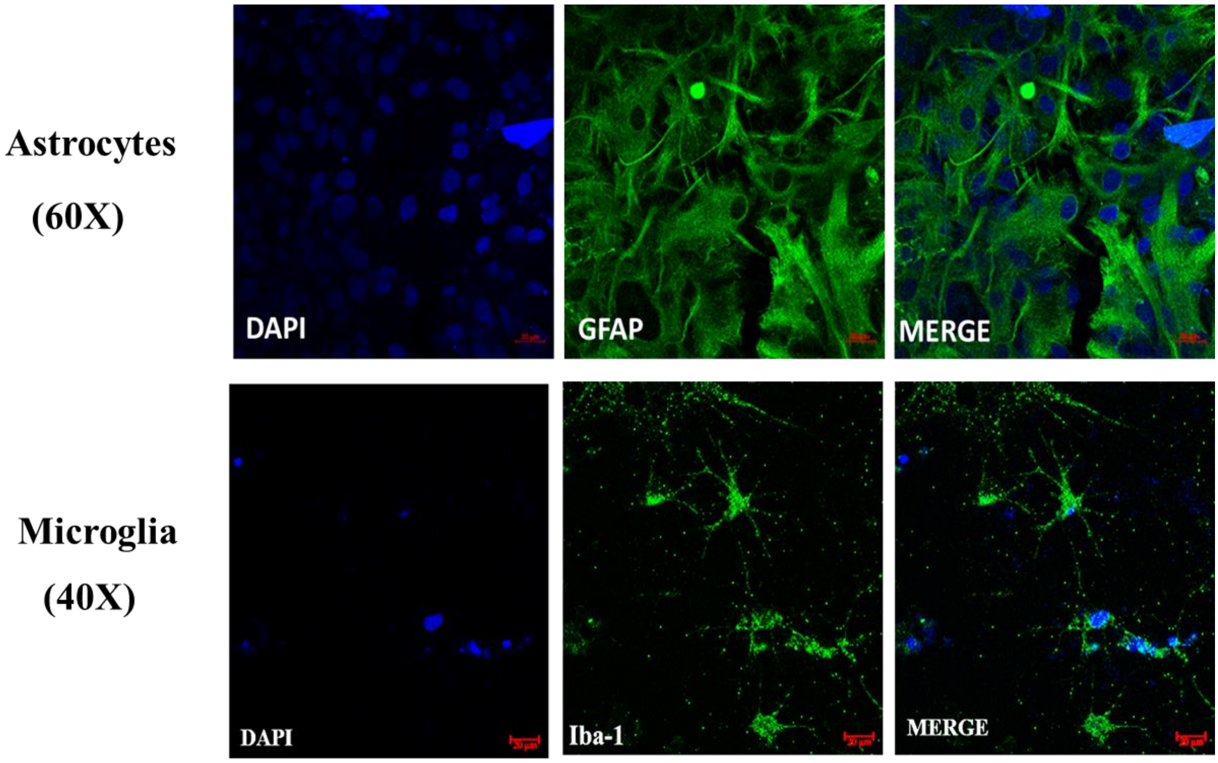
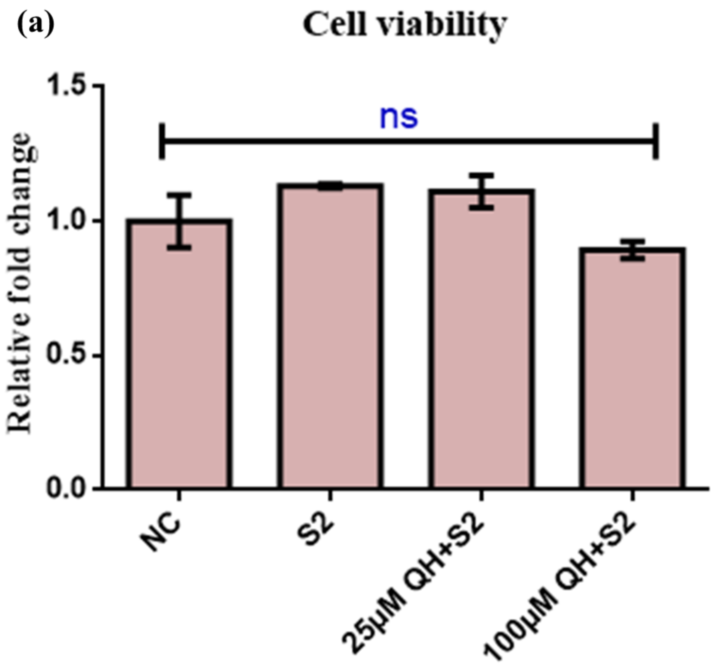


Fig.3.3 Cell viability and morphological analysis of quercetin treated and MeCP2 knock-down astrocytes (a) Cell viability was assessed by MTT assay. Graph shows no fold change in viability relative to control (NC). Data represented as mean+SEM (n=4) (b) Phase contrast images (20X magnification) after quercetin and MeCP2 siRNA incubations. Only 100µM quercetin(QH) treated astrocytes show minor change in morphology compared to control.

(a)


Cell viability
Relative fold change
1.5
1.0
0.5
0.0
ns
NC
S2
25µM QH+S2
100µM QH+S2

(b)

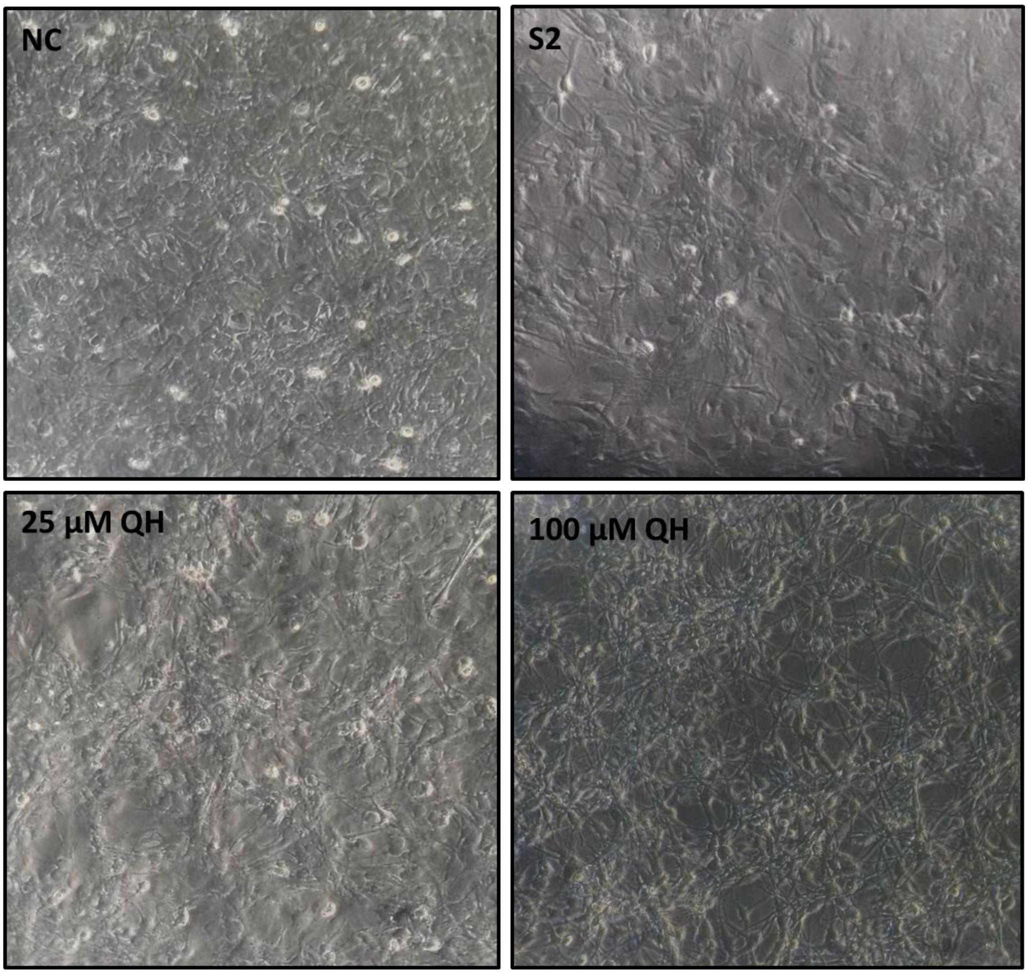

NC
S2
25 µM QH
100 µM QH

Fig.3.4 Effect of quercetin on mitochondrial respiratory chain genes expression in MeCP2 knock-down astrocytes Quantitative and semi-quantitative RT-PCR was performed to analyse relative mRNA levels in quercetin treated and untreated MeCP2 siRNA transfected astrocytes.(a) Representative image of band intensities. (b-g) Graphs for MeCP2, GFAP, Uqcrc1, Ndufv2 and BDNF genes 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)

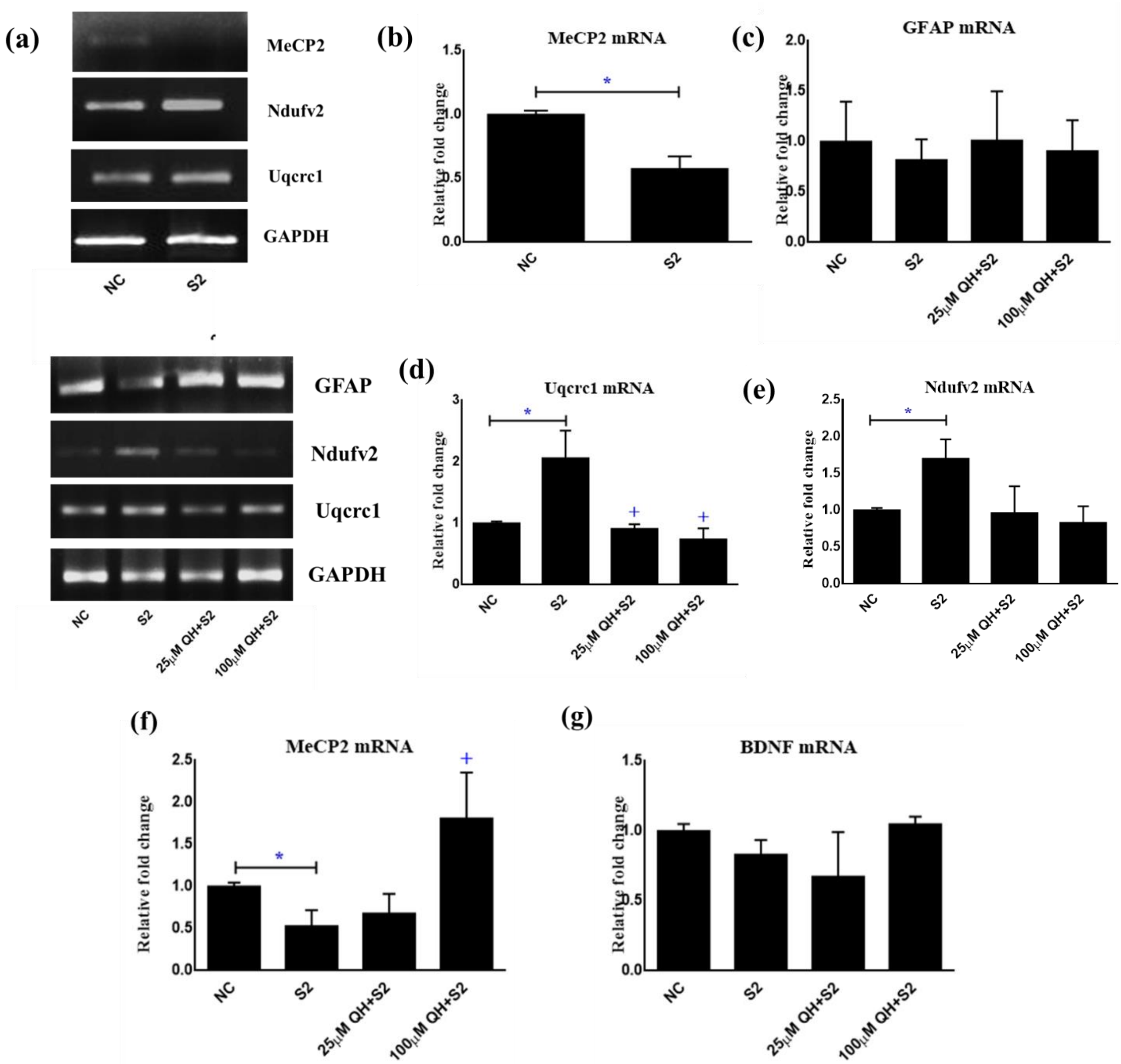


Fig. 3.5 Effect of quercetin on mitochondrial respiratory chain protein expression in MeCP2 knock-down astrocytes Western blot analysis was performed to analyse relative protein levels in quercetin treated and untreated MeCP2 siRNA transfected astrocytes.(a,b) Representative image of band intensities of MeCP2, Ndufv2 and β-actin as internal control. (c) Graph for MeCP2 (d) Graph for Ndufv2 proteins 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 proteins expression as compare to MeCP2 deficient astrocytes

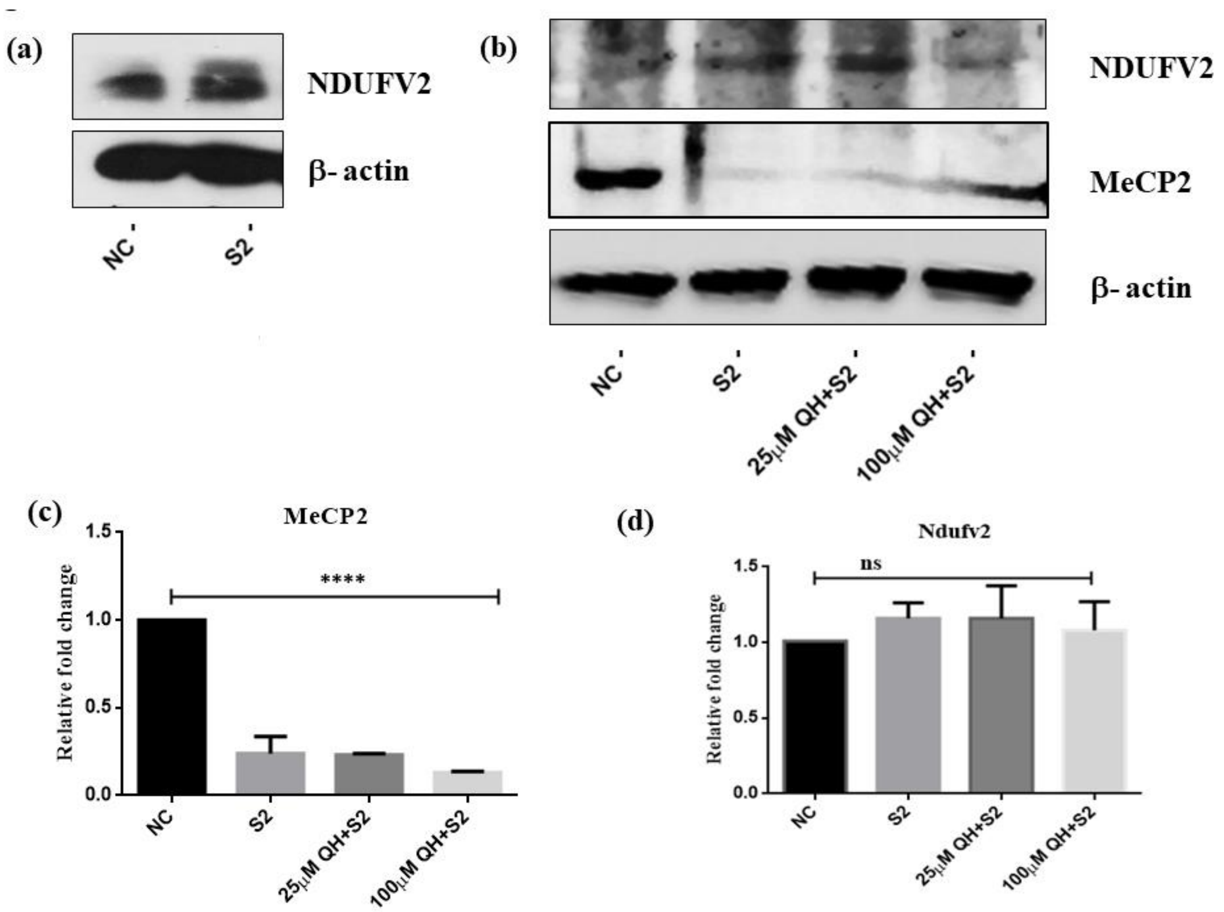


Fig. 3.6 Effect of quercetin on mitochondrial respiratory chain enzyme activities in MeCP2 knock-down astrocytes Spectrophotometric analysis revealed significant difference in MeCP2 siRNA transfected and quercetin treated MeCP2 transfected astrocytes. (a-d) Graph for complex-I, II, III and IV enzyme activities. Data represent mean specific activity in nmole/min/mg 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)

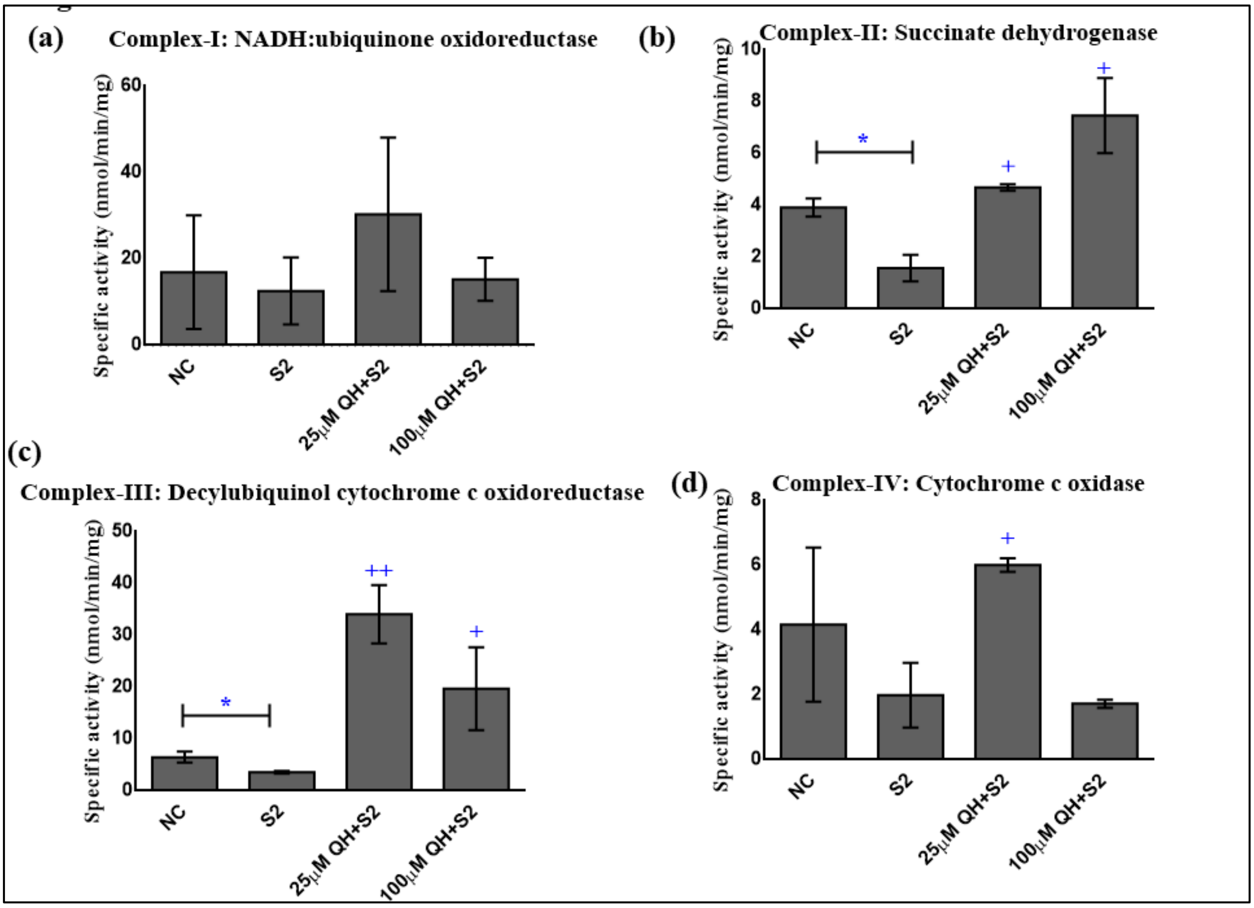


Fig. 3.7 Effect of quercetin on ROS production in MeCP2 knock-down astrocytes Cells after 24 hours MeCP2 siRNA transfection were incubated for 30min with H2.DCF.DA fluorescent dye. Graph for ROS production depicts no change in ROS production in MeCP2 knock-down (S2) or quercetin treated (QH+S2) groups. Data is represented as mean relative fluorescence unit± SEM (RFU) (n=4)

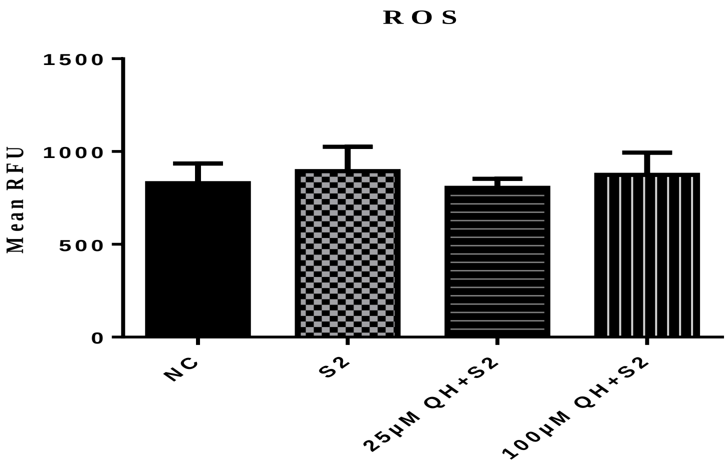


Fig. 3.8 Effect of quercetin on intracellular calcium in MeCP2 knock-down astrocytes Intracellular calcium was measured by fluorometric analysis of calcium binding fluorescent dye Cal520AM. Graph for intracellular calcium shows significant differences. Data is represented as mean relative fluorescence 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)

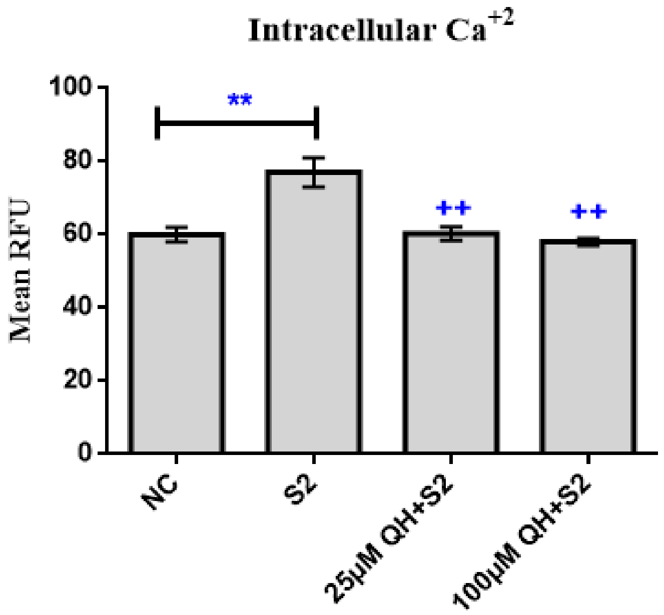


Fig. 3.9 Effect of quercetin on mitochondrial membrane potential in MeCP2 knock-down astrocytes Mitochondrial membrane potential was measured by fluorometric analysis of cationic dye-Rhodamine123 in non-quenching mode. Graph shows significant differences. Data is represented as mean relative fluorescence 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).

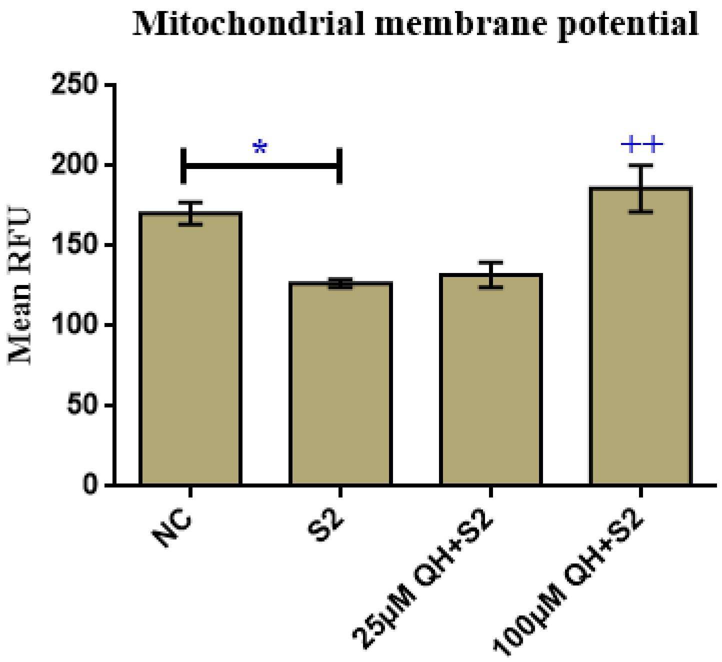
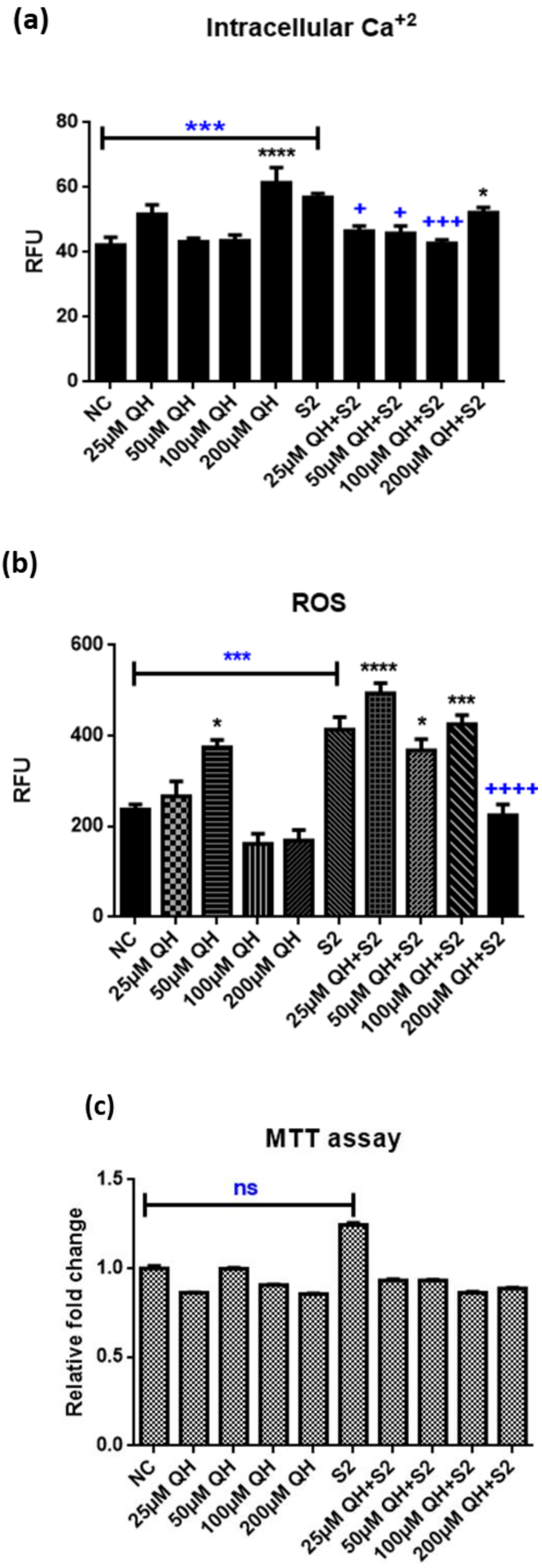


Fig.3.10 Analysis of intracellular calcium, ROS and cell viability in QH treated MeCP2 knock down C6 glial cell line After quercetin (QH) pre-incubation the MeCP2 siRNA transfection was carried out followed by fluorometric measurements. This image depicts graphical representation of relative fluorescence of (a) Intracellular [Ca^{+2}] (b) ROS production (c) Cell viability measured by MTT assay. QH treated groups were compared to negative control (NC) and MeCP2 knock-down. Data represented as mean $\pm$ SEM from four independent experiments. p values shown with asterisks (*p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001) are compared to negative control whereas, p value shown in blue color plus sign ($^{+}$P < 0.05, $^{++}$P < 0.01 $^{+++}$P < 0.001, $^{++++}$P < 0.0001) are compared to MeCP2 knock-down (S2)



(a)
Intracellular Ca+2
RFU
0
20
40
60
80
***
****
+
+
+++
*
NC
25µM QH
50µM QH
100µM QH
200µM QH
S2
25µM QH+S2
50µM QH+S2
100µM QH+S2
200µM QH+S2
(b)
ROS
RFU
0
200
400
600
***
*
****
*
***
++++
NC
25µM QH
50µM QH
100µM QH
200µM QH
S2
25µM QH+S2
50µM QH+S2
100µM QH+S2
200µM QH+S2
(c)
MTT assay
Relative fold change
0.0
0.5
1.0
1.5
ns
NC
25µM QH
50µM QH
100µM QH
200µM QH
S2
25µM QH+S2
50µM QH+S2
100µM QH+S2
200µM QH+S2

3.4 Summary

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 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 signalling 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 or phenotypic expression in Rett syndrome is still in grey area. Also, whether the mitochondrial impairment is solely by direct involvement of MeCP2 in mitochondrial genes transcription or indirect effects due to dysregulated phosphorylation of signalling 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 genes expressions (at a given time).

Quercetin, a well-known anti-oxidant has been explored in many of the diseases involving mitochondria but observations from the present study 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 genes expression with minor up-regulated Ndufv2 protein expression in MeCP2 knock-down astrocytes. In spite of the up-regulated genes 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 genes expressions are not directly co-related 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 genes expression but increased MRC enzyme activities compare to MeCP2 knock down alone that indicate quercetin's multiple targets to ameliorate the imbalance in context-dependent manner. Studies in C6 glial cells also show beneficial modulatory effects of QH in MeCP2 deficient condition.

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 till date, improving the downstream parameters might manifest the slowed progression or symptoms of the diseases. Furthering our understanding by more observations and advanced methods will throw more light upon the pathogenesis of Rett syndrome in regards to mitochondrial dysfunction in glial cells.