Chapter-IV

To determine the effects of docosahexaenoic acid (DHA) on mitochondrial dysfunction mediated by MeCP2 deficiency in astrocytes

4.1 Introduction

As discussed in Chapter-III, mitochondrial dysfunction has been observed in MeCP2 null mice and Rett syndrome patients and has been demonstrated to be playing an important role in disease progression (Kriaucionis et al. 2006, Gold et al. 2014, Valenti et al. 2017).

Docosahexaenoic acid (DHA), an omega-3 unsaturated fatty acid, is a component of plasma membrane as well as mitochondrial membrane (Watkins, Carter, and German 1998, Ting, Chao, and Hsu 2015, Hashimoto et al. 1999, Seo et al. 2006, Shaikh and Brown 2013). DHA has been reported to possess neuroprotective role (Blondeau et al. 2002, Lauritzen et al. 2000). Some of the reports show anti-oxidant effects of DHA (Kusunoki et al. 2013, Wu, Ying, and Gomez-Pinilla 2004), whereas others show pro-oxidant effects of DHA (Watkins, Carter, and German 1998, Li et al. 2010, Hsu and Yin 2016, Di Nunzio, Valli, and Bordoni 2016) and taking together recent studies it appears that DHA function is of paradoxical nature. Surprisingly, in the last decade none of the studies has focused on DHA's effect on ATP synthesis. Moreover, there are very few reports on DHA's direct effects on mitochondrial respiratory chain enzymes. In DHA and DHA rich diet fed rodent models, electron transport chain activities were found to be reduced in cardiac and liver tissues (Sullivan et al. 2018, Aoun et al. 2012) whereas, electron transport chain complexes III and IV activities were significantly increased in skeletal muscle tissue in rodent model (Le Guen et al. 2015).

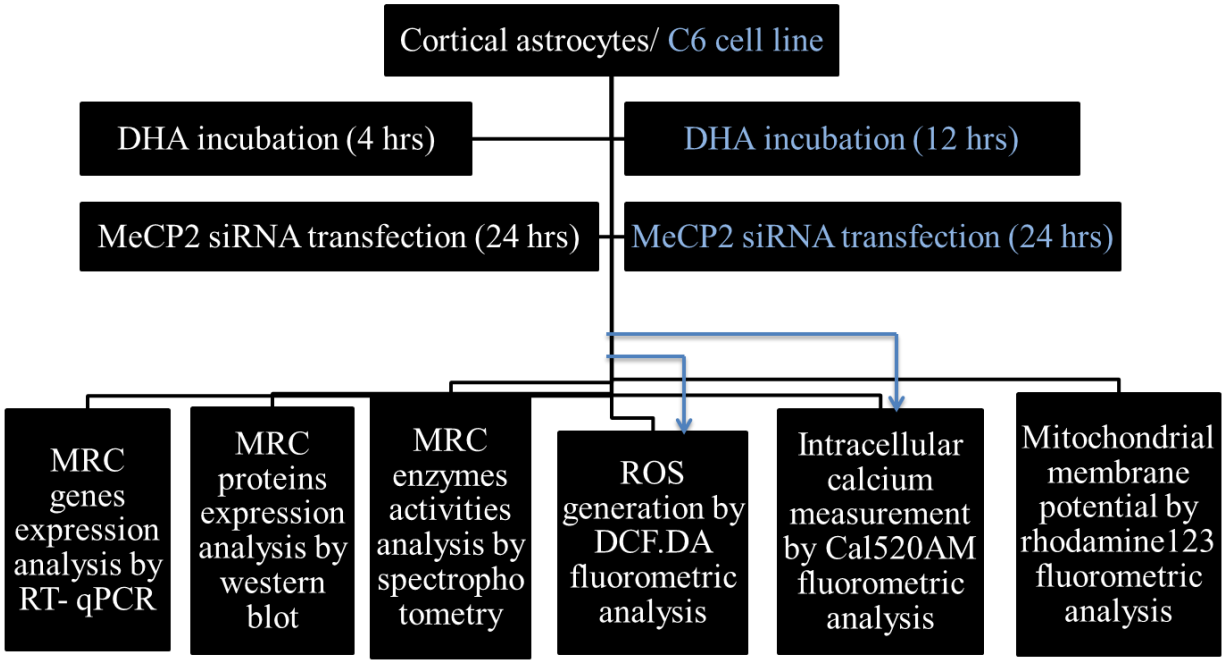
DHA has been reported to modulate calcium levels as well (Begum et al. 2012, Sergeeva, Strokin, and Reiser 2005). DHA suppresses thrombin-evoked Ca^{2+} response, inhibits store-operated Ca^{2+} entry, and ATP-mediated intracellular Ca^{2+} oscillations in rat astrocytes (Sergeeva, Strokin, and Reiser 2005). DHA inhibits the exogenous IP_3-mediated $Ca^{2+}{}_{ER}$ release rate directly in ischemic condition (Begum et al. 2012). It has been shown to inhibit voltage-gated Na^{+} channels,

high voltage-activated Ca^{2+} channels in hippocampal CA1 neurons (Vreugdenhil et al. 1996) and T-type Ca^{2+} currents in bovine AZF cells at micromolar concentrations (Danthi, Enyeart, and Enyeart 2005). Moreover, DHA supplementation has been shown to enhance gap junction coupling capacity in rat cortical astrocytes (Champeil-Potokar et al. 2006) which could further influence glial communication by Ca^{2+} waves through gap junctions. DHA has also been observed to normalize or increase BDNF in disease models (Fang et al. 2017, Wu, Ying, and Gomez-Pinilla 2004, Balanza-Martinez et al. 2011). Mature BDNF binds to the TrkB receptors on plasma membranes and activates downstream signalling pathways involving PLC-γ, ERK/MAPK and Akt/PKB. In brief, activation of PLC-γ leads to the release of calcium from the ER which leads to the activation of CaMKII that ultimately causes phosphorylation of CREB transcription factor; activation of the ERK/MAPK pathway leads to the phosphorylation of CREB; and activation of PI3K leads to phosphorylation of Akt/PKB and mTOR which regulate translation initiation. Thus, increased BDNF through DHA has the potential to regulate intracellular signalling pathways, transcription factors and ultimately the transcription of genes (Balanza-Martinez et al. 2011).

Beneficiary effects of DHA in Rett syndrome patients (MeCP2 mutations) have also been reported. Oxidative stress marker (F2-isoprostanes (F2-IsoPs) was found to be significantly increased in Rett patients, which was ameliorated in docosahexaenoic acid (DHA) plus eicosapentaenoicacid (EPA)-omega-3 polyunsaturated fatty acids (PUFAs) fed patients. Also the clinical symptoms were found to be reduced in omega-3 fatty acids treated Rett patients (Leoncini et al. 2011). Red blood corpuscles (RBCs) play a major role in pulmonary gas exchanges and the alteration in their morphology suggest imbalance in the oxidative stress parameters. In a study on RBCs from Rett patients, the altered RBCs morphology was found to be partially rescued by Omega-3 PUFAs (DHA plus EPA) and the oxidative stress markers were also reduced in Omega-3 PUFAs treated Rett patients (Ciccoli et al. 2012). Another study in Rett syndrome patients, show partial rescue of clinical symptoms and reduced oxidative stress markers in Omega-3 PUFAs given patients (De Felice et al. 2012).

Although, the previous reports have documented anti-oxidant property of DHA along with EPA in Rett syndrome patients (with classical MeCP2 mutations), the molecular mechanism underlying the effects of DHA in MeCP2 deficient glial cells has not been evaluated. In the current study, the DHA (25μM, 100 μM) pre-incubated astrocytes were transfected with MeCP2 siRNA and mitochondrial respiratory chain(MRC) genes, proteins, enzyme activities, ROS, intracellular calcium, mitochondrial membrane potential and cell viability were assessed. Further, intracellular calcium, ROS and cell viability were also ascertained in MeCP2 deficient C6 glial cells.

4.2 Experimental design



4.3 Results and Discussion

4.3.1 Effect of DHA on cell viability & morphology of cortical astrocytes

To analyse the effect of DHA (25,100µM) on cell survival, MTT assay was performed. DHA protected MeCP2 knock-down astrocytes in a dose dependent manner. DHA (25 µM) treatment showed protective effect whereas, 100µM DHA treatment showed cytoxicity in MeCP2 knock-down astrocytes. In phase contrast image analysis, no change in morphology was observed in MeCP2 knock-down and 25µM DHA treated MeCP2 knock-down astrocytes but subtle difference in morphology was observed in 100 µM DHA treated MeCP2 knock-down astrocytes (Fig.4.1).

4.3.2 DHA restores mitochondrial respiratory chain complex-III (Uqcrc1) but does not affect Complex-I (Ndufv2) mRNA levels

In this study, quantitative RT-PCR was performed for Uqcrc1, Ndufv2, GFAP and BDNF genes. Uqcrc1 gene expression was normalized in DHA (25, 100 µM) treated MeCP2 knock-down astrocytes (S2).However, Ndufv2 gene expression did not change in DHA treated MeCP2 knock-down cells in comparison to untreated MeCP2 knock-down cells. Interestingly, previous studies

have shown potential of DHA in regulating MRC genes and transcription factors involved in MRC gene expression and regulation. For instance, significantly increased mtDNA copy number, up-regulated mRNA levels of PGC-1α, NRF1, and Tfam transcription factors involved in mitochondrial biogenesis and up-regulated PGC-1α promoter activity in DHA(50μM) treated mouse C_2C_{12} myoblasts were observed (Lee et al. 2016). Increased Nrf1 and PGC-1α transcription factors gene expression and up-regulated genes for mitochondrial respiratory chain complexes (i.e. mt-Co1, mt-Co3, mtNd1, mt-Nd4, mt-Atp6 and mt-Atp8) were observed in white fat tissue from mice fed with DHA contained high-fat diet compared to only high-fat diet fed controls (Flachs et al. 2005). PGC-1α, NRF1 and Tfam mRNAs expressions were maintained in mice models fed with fish oil (containing DHA) + high-fat diet compared to their expressions in only high-fat diet fed mice (Lanza et al. 2013). PGC-1α gene expression was also found to be increased in omega-3 fatty acids (DHA and EPA) in human skeletal muscles cell line (Vaughan et al. 2012, Lanza et al. 2013). Contradictory to the previous reports, the mRNA expression of ERRα (estrogen-related receptor alpha), Tfam, and PGC1α were downregulated in C2C12 myoblasts treated with differentiation medium that contained fatty acids (50-μM EPA and DHA)(Hsueh, Baum, and Huang 2018). However, this is the first report showing MeCP2-transcription factor modulation by DHA.

In the current study, data also show significantly increased MeCP2 and BDNF genes expression in 100μM DHA treated MeCP2 knock-down astrocytes (Fig.4.2). Intriguingly, some studies suggest that BDNF participates in a range of metabolic events, including glucose utilization and energy management (Yamanaka et al. 2007, Gomez-Pinilla, Vaynman, and Ying 2008). Studies in Neuro-2a (N2a)-neuroblastoma cells showed increased PGC-1α protein expression in 7,8-dihydroxyflavone (7,8-DHF), a TrkB (BDNF receptor) treated cells (Agrawal et al. 2014).

Overall, up-regulated MeCP2 directly or increased BDNF (gene regulated by MeCP2) by DHA could be responsible for Uqcrc1 normalized expression. Ndufv2 gene expression did not normalize in DHA treated MeCP2 knock-down astrocytes which could be due to the differential regulation of transcription factors involved in mitochondrial respiratory chain genes by DHA as documented in previous reports. It also could be due to the cell specific action as DHA might not be incorporating in same numbers in mitochondrial phospholipids of all the cells.

4.3.3 DHA increases MeCP2 protein expression but does not lower Ndufv2 expression in DHA treated MeCP2 knock-down astrocytes

In protein expression western blot analysis, MeCP2 expression was found to be increased in 100μM DHA treated MeCP2 knock-down astrocytes compared to negative control that is consistent with MeCP2 mRNA level (Fig.4.3). Ndufv2 protein expression was also increased in 100μM DHA+S2 group compared to negative control which too is in line with Ndufv2 mRNA level. Though, MeCP2 mRNA and protein expressions were increased in 100μM DHA+S2 astrocytes, the Ndufv2 did not decrease which indicate that there could be other regulators of Ndufv2 expression through which DHA acts.

DHA in skeletal muscles did not show change in mitochondrial respiratory chain complexes proteins expression compared to control in rodent model(Le Guen et al. 2015) However, immunoblotting analysis revealed that the biosynthesis of nuclear-encoded mitochondrial proteins (COX6, SDHA and ATP5A1) is also upregulated (Flachs et al. 2005). In other studies, COII (ETC protein), TFAM and PGC-1α proteins have been observed to be reduced under the n−3 deficiency while the adequate levels of dietary n−3 fatty acid maintained the levels of proteins after Traumatic brain injury (Agrawal et al. 2014).

4.3.4 DHA up-regulates mitochondrial respiratory chain enzyme activities in MeCP2 knock down astrocytes

In spectrophotometric analysis, mitochondrial respiratory chain complexes – I, II and III enzyme activities were found to be substantially increased in 100μM DHA+ S2 astrocytes in comparison to MeCP2 knock-down alone and negative control (Fig.4.4). This implies DHA potential in modulating ETC enzymes activities independent of mitochondrial damage.

DHA incorporated in cardiolipin was found to be lowering mitochondrial respiratory chain complexes- I, IV, V, and I+III activities in mice fed with western diet with DHA (Sullivan et al. 2018). In polarographic measurement decreased mitochondrial bioenergetic functions(decrease in respiratory control index) and decreased ATP levels associated with increasing DHA levels in mitochondrial membranes in mice has been observed (Stillwell et al. 1997). Mice fed HFD with fish oil (containing DHA) showed no change in mitochondrial abundance and respiratory capacity compared with only HFD fed mice controls (Lanza et al. 2013). In C2C12 myoblasts during skeletal muscle differentiation, decreased respiration rate was observed (Hsueh, Baum, and Huang 2018).

4.3.5 DHA lowers intracellular calcium level in MeCP2 knock down astrocytes

In intracellular calcium measurement by fluorometry, reduced $[Ca^{2+}]_i$ in 100μM DHA treated MeCP2 knock-down astrocytes were found compared to untreated MeCP2 knock-down and negative control both. Thus, DHA lowers $[Ca^{2+}]_i$ in context (disease severity)- independent manner.

4.3.6 DHA does not alter ROS in MeCP2 knock down astrocytes

In DCF.DA fluorometric analysis, no changes in ROS production in DHA treated MeCP2 knock-down cells were observed. Mitochondrial electron transport chain is a main source for ROS production but in spite of the alteration in ETC complexes activities, the ROS levels were found unchanged in astrocytes (Fig.4.6). Similarly, in fish oil (containing DHA) treated *in vivo* model , the H2O2 production was same as controls (Lanza et al. 2013).

4.3.7 DHA does not modulate mitochondrial membrane potential in MeCP2 knock down astrocytes

Despite of DHA's modulatory effect on mitochondrial respiratory chain complexes activities in MeCP2 knock-down cells, there was no increase in mitochondrial membrane potential. The DHA treated MeCP2 knock-down cells were also seen in depolarized state like untreated MeCP2 knock-down astrocytes. This could be due to H^+ or other positive ions leakage in DHA treated cells which did not allow accumulation of cationic dye in mitochondria and showed depolarized state. This phenomenon could be independent of mitochondrial respiratory chain complexes activities.

Previous report shows that upon incorporation of DHA into mouse mitochondria, a marked decrease in respiratory rate, increase in H^+ movement and decrease in membrane potential occurs (Stillwell et al. 1997). The data from spectrophotometric electron transport chain complexes activities analysis and from fluorometric mitochondrial membrane potential are contradictory and needs further investigation.

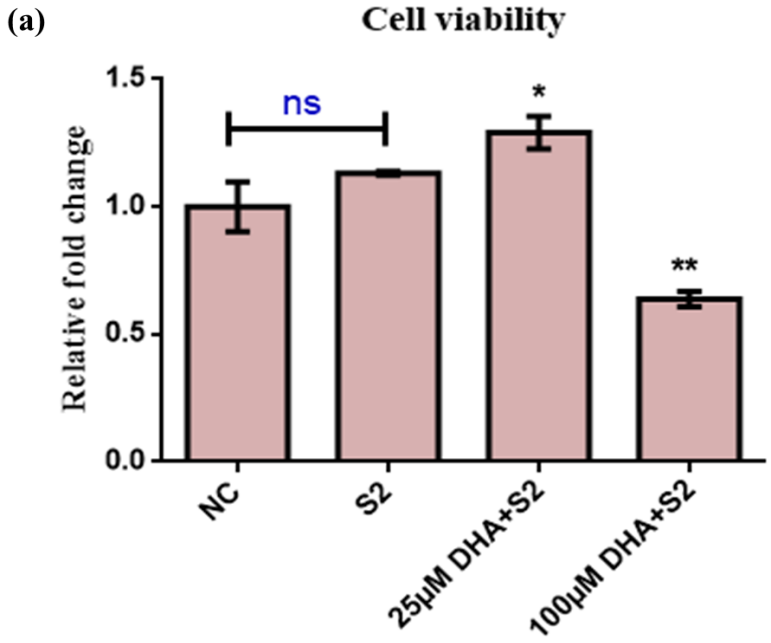
4.3.8 DHA changes intracellular calcium, ROS levels in dose dependent manner in MeCP2 knock-down C6 glial cells

DHA (25, 50, 100 μM) showed increased $[Ca^{2+}]_i$ in MeCP2 knock-down cells as well as control and decreased $[Ca^{2+}]_i$ in DHA (200 μM) treated cells. Thus, DHA behaved in a context-independent manner.

ROS level robustly increased in DHA (25, 50 μM) treated MeCP2 knock-down cells compared to negative control and MeCP2 knock-down(S2) cells. It was unchanged in DHA (25, 50,100, 200 μM) treated negative control and DHA (100, 200 μM) treated MeCP2 knock-down cells. Thus, DHA acted as pro-oxidant in C6 glioma cell line. Study in HT-29 (Human colonic adenocarcinoma) cell line has shown increased oxidant production by accumulating in Cardiolipin (Watkins, Carter, and German 1998). In the same study, mitochondrial membrane potential was also found to be increased in DHA treated cells (Watkins, Carter, and German 1998).

In cell viability assay also negligible effect of DHA was observed (Fig.4.8). DHA has already been demonstrated to partially rescue Rett syndrome in patients, which is attributed to its anti-oxidant effects (De Felice et al. 2012), but surprisingly, we did not observe DHA beneficial effects in MeCP2 deficient glial cells and this could be due to cell type or dosage used in the present study.

Fig.4.1 Cell viability analysis of DHA treated and MeCP2 knock-down astrocytes Cell viability was assessed by MTT assay. Graph shows 0.29 fold increase in viability in 25µM DHA+S2 treated and 0.36 fold decrease in viability in 100µM DHA+S2 treated astrocytes relative to control(NC).Data represented as mean+SEM (n=4) (b) Phase contrast images taken after MeCP2 siRNA incubation (24hr) and DHA (4hr) incubation.



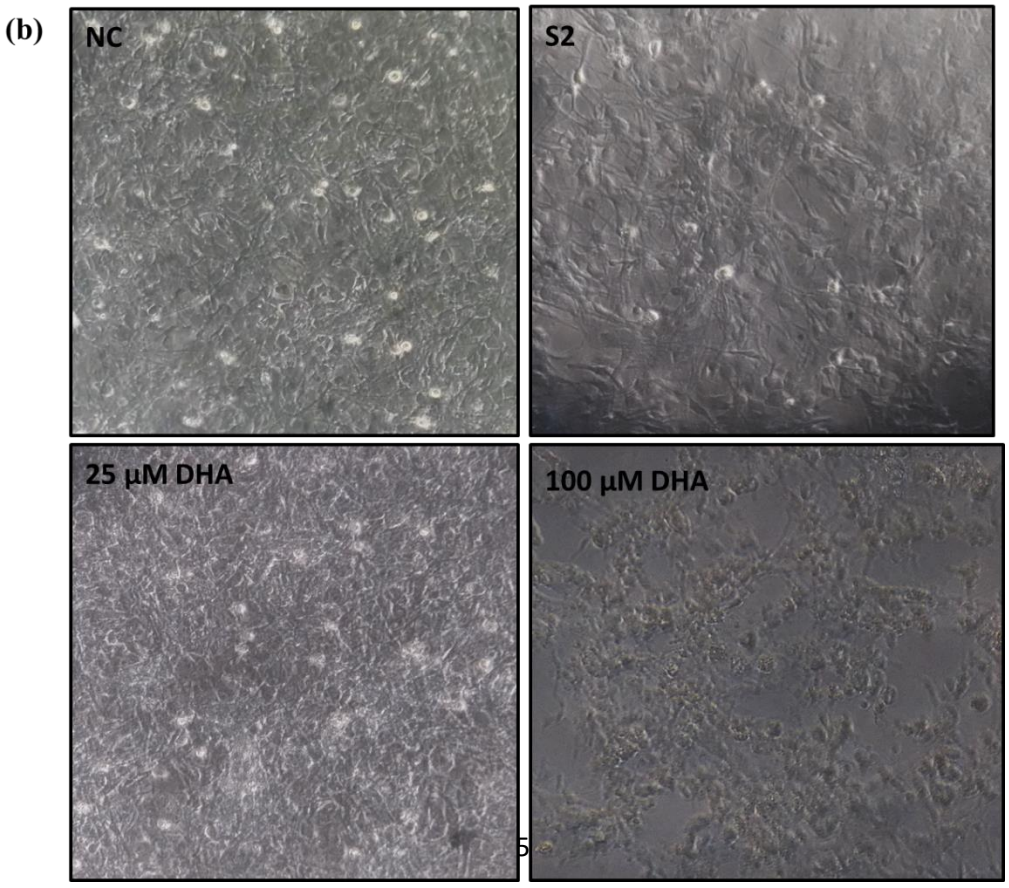


Fig.4.2 Effect of DHA on mitochondrial respiratory chain genes expression in MeCP2 knock-down astrocytes Quantitative RT-PCR was performed to analyse relative mRNA levels in DHA treated and untreated MeCP2 siRNA transfected astrocytes. (a-e) Graphs for MeCP2, GFAP, Uqcrc1, Ndufv2 and BDNF genes expression normalized to GAPDH and compared with control. Data are presented as mean ± SEM (n=4). p value indicated with asterisks (*P < 0.05,**P < 0.01) 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)

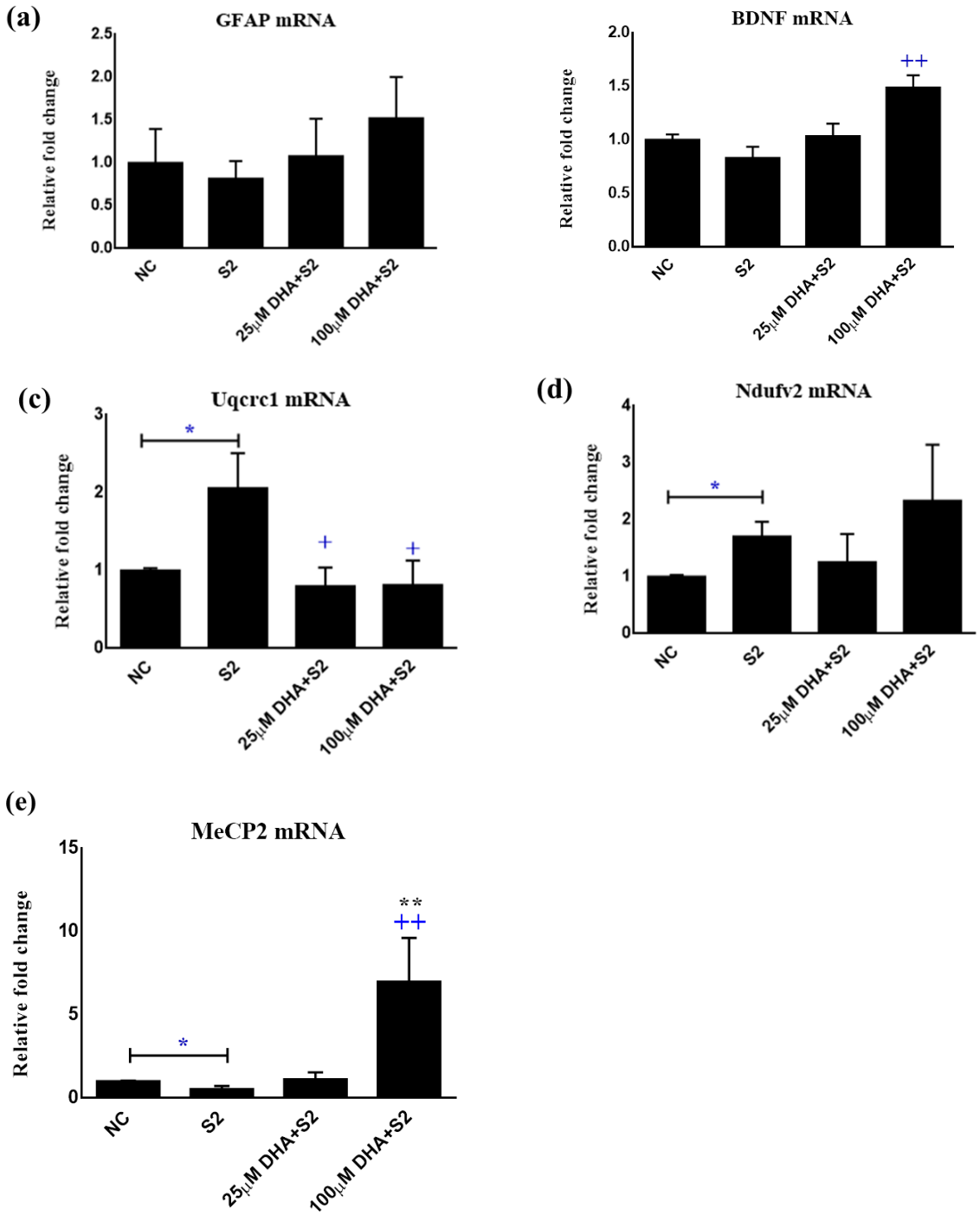


Fig.4.3 Effect of DHA on mitochondrial respiratory chain protein expression in MeCP2 knock-down astrocytes Western blot analysis was performed to analyse relative protein levels in DHA treated and untreated MeCP2 siRNA transfected astrocytes.(a) Representative image of band intensities of MeCP2, Ndufv2 and β-actin as internal control. (b) Graph for MeCP2 (c) Graph for Ndufv2 proteins expressions normalized to β-actin and compared with control. Data are presented as mean ± SEM (n=3); p value indicated with asterisks (*P <0.05,**P < 0.01) and ns (non-significant) are in comparison to negative control (NC).

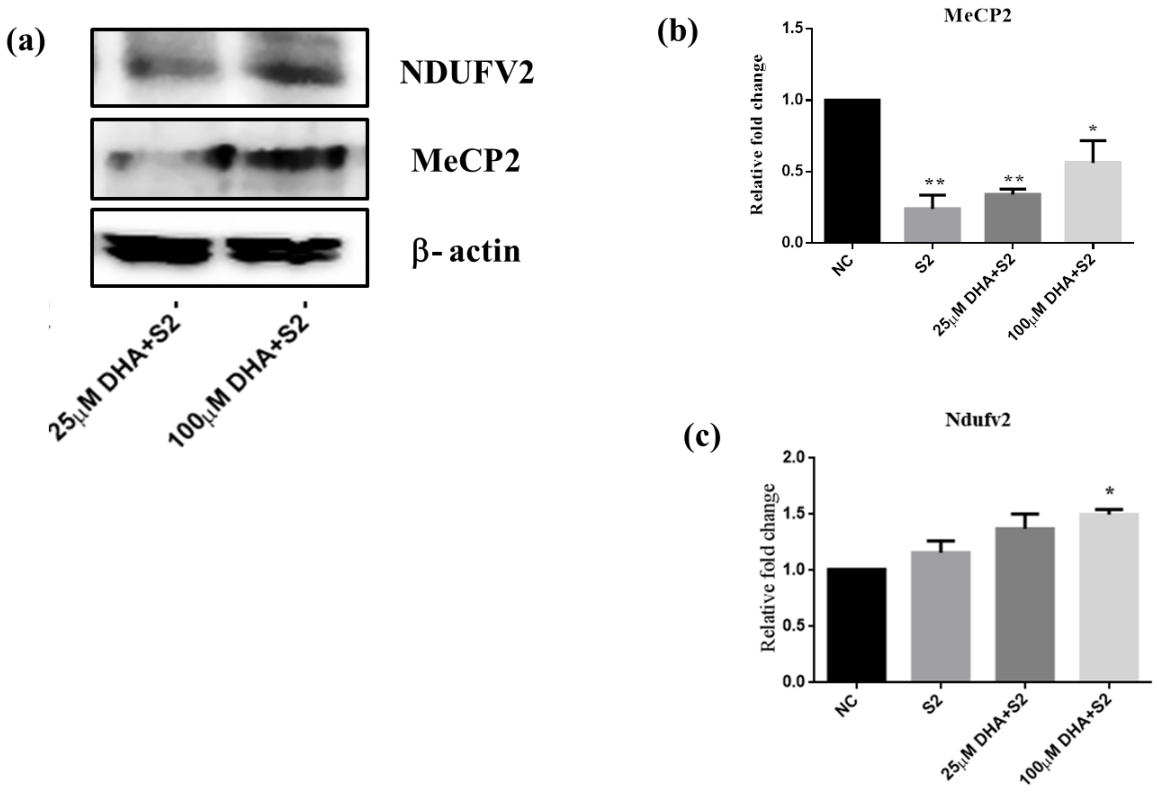


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Fig.4.4 Effect of DHA on mitochondrial respiratory chain enzyme activities in MeCP2 knockdown astrocytes. Spectrophotometric analysis revealed significant difference in MeCP2 siRNA transfected and DHA treated MeCP2 transfected astrocytes compared to negative control(NC) and MeCP2 knock-down alone(S2) respectively. (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)

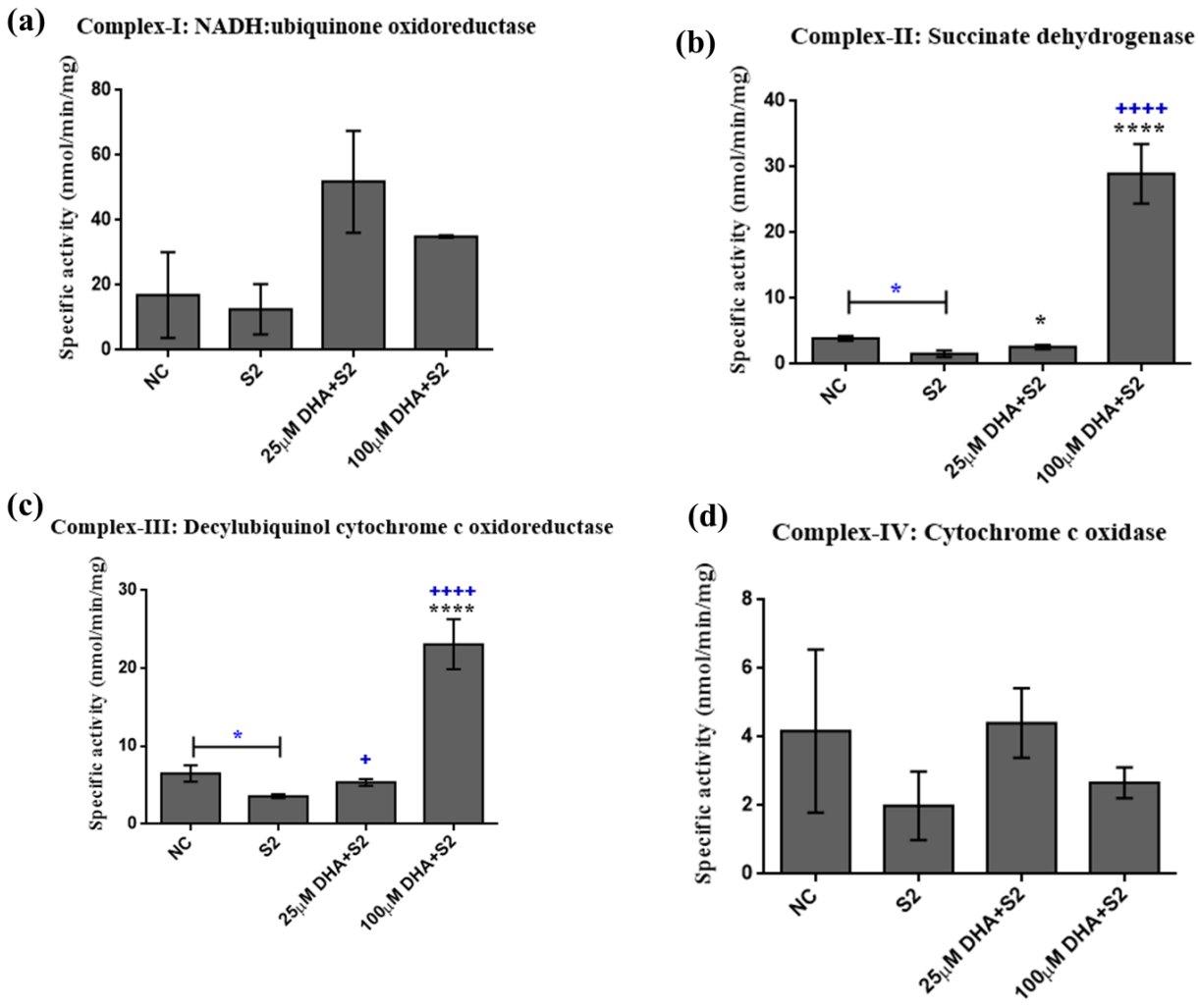


Fig.4.5 Effect of DHA on ROS production in MeCP2 knock-down astrocytes Cells after 24 hours MeCP2 siRNA transfection were incubated for 30min with H2.DCF.DA fluorescent dye. Graph for ROS production depicts no change in ROS production in MeCP2 knock-down (S2) or DHA treated (DHA+S2) groups. Data is represented as mean relative fluorescence unit ± SEM (RFU) (n=4)

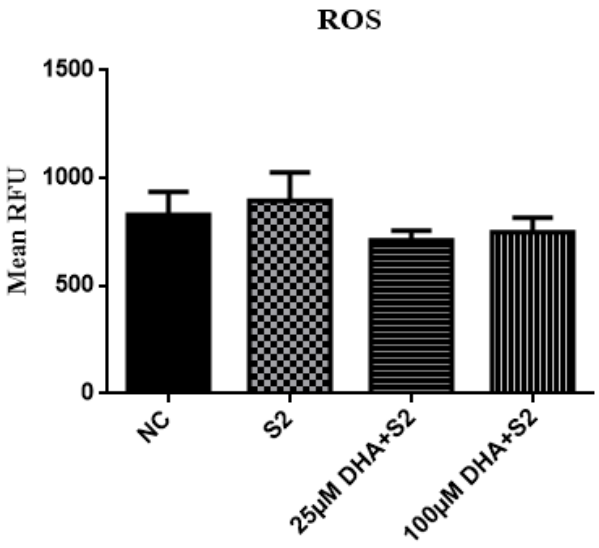


Fig.4.6 Effect of DHA on intracellular calcium in MeCP2 knock-down astrocytes Intracellular calcium was measured by fluorometric analysis of calcium binding fluorescent dye Cal520AM. Graph for intracellular calcium shows significant differences. Data is represented as mean relative fluorescence unit (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)

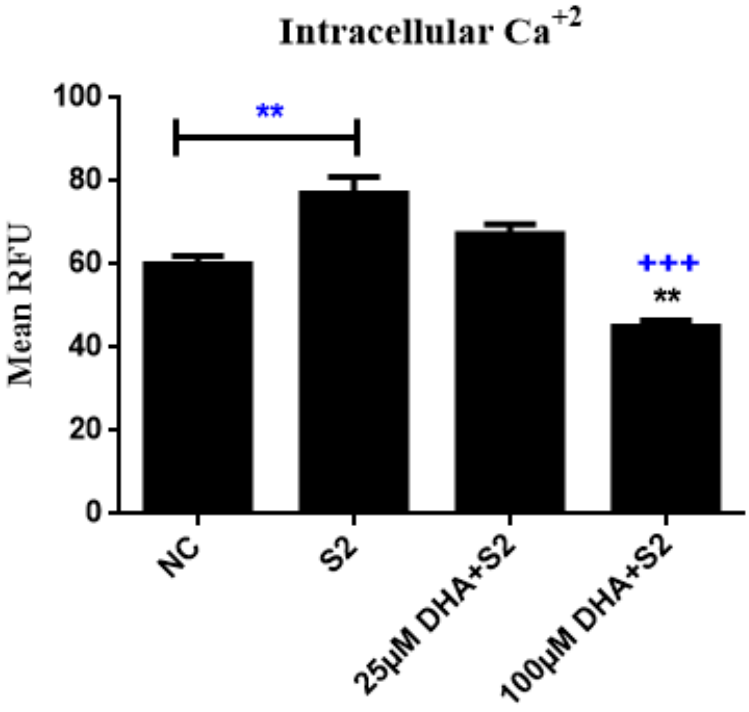


Fig.4.7 Effect of DHA on mitochondrial membrane potential in MeCP2 knockdown astrocytes mitochondrial membrane potential was measured by fluorometric analysis of cationic dye Rhodamine123 in non-quenching mode. Graph shows significant differences. Data is represented as mean relative fluorescence unit (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)

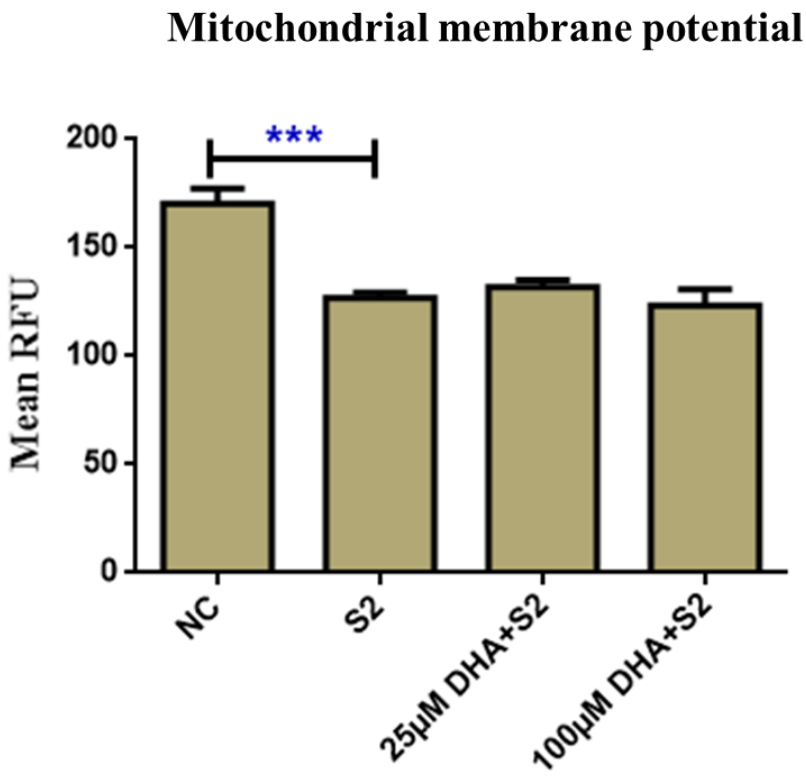
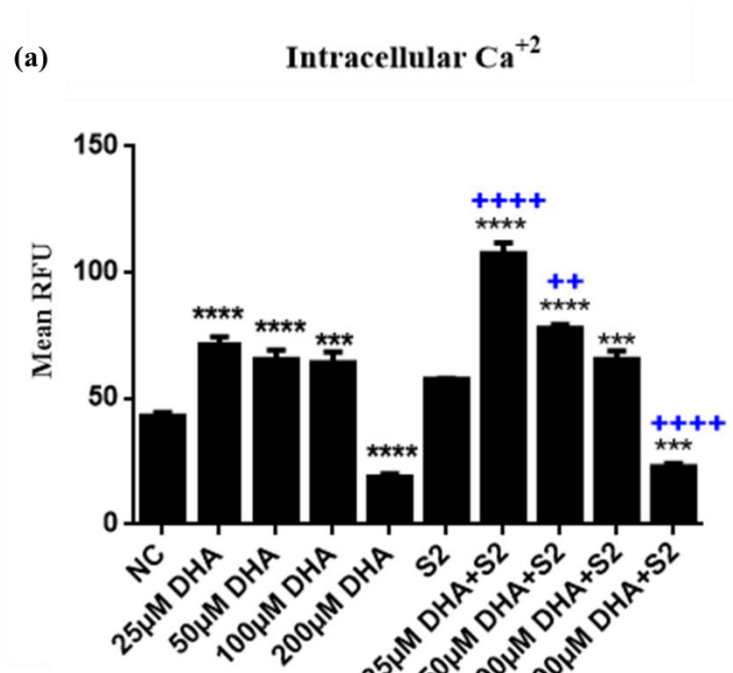


Fig.4.8 Analysis of intracellular calcium, ROS and cell viability in DHA treated MeCP2 knock down C6 glial cell line After DHA pre-incubation the MeCP2 siRNA transfection was carried out followed by fluorometric measurements. This image depicts graphical representation of relative fluorescence of **(a)** Intracellular [Ca^{2+}] **(b)** ROS production **(c)** Cell viability measured by MTT assay. DHA treated groups were compared to negative control (NC) and MeCP2 knock-down. Data represented as mean ± 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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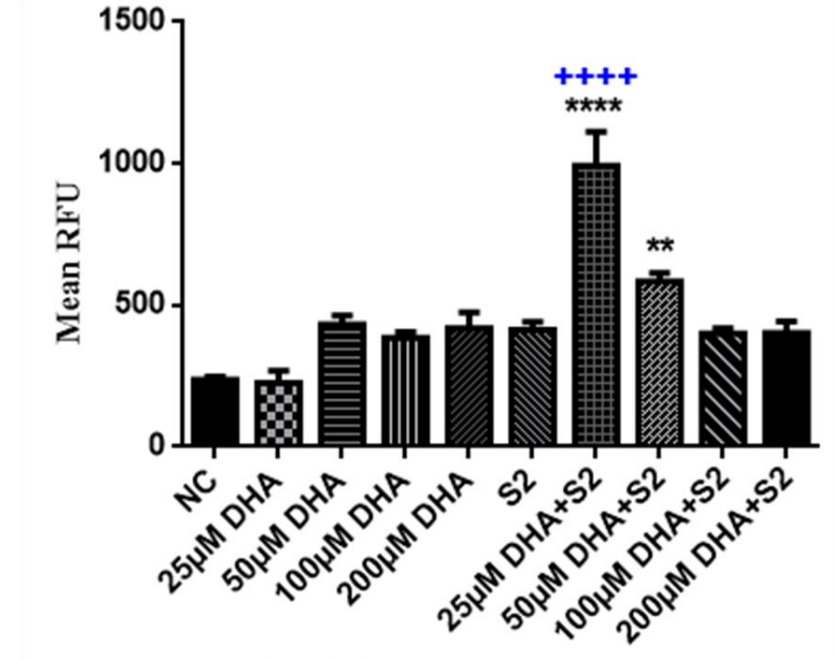
(b)
Mean RFU
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25μM DHA
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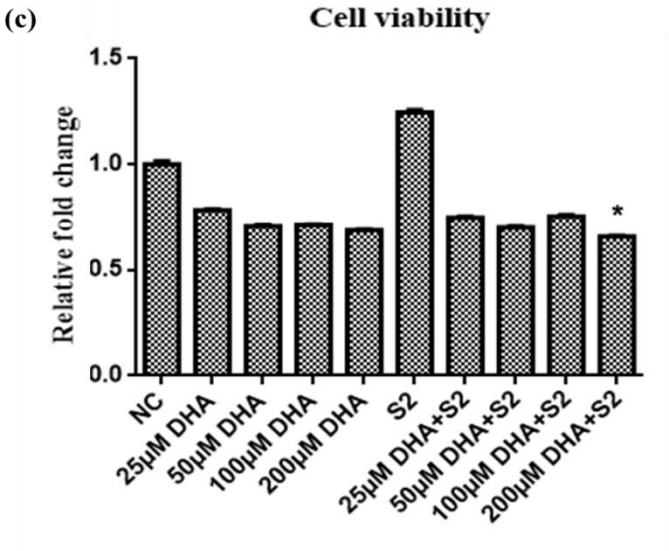
200μM DHA+S2


(c)
Cell viability
Relative fold change
1.5
1.0
0.5
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NC
25μM DHA
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25μM DHA+S2
50μM DHA+S2
100μM DHA+S2
200μM DHA+S2

4.4 Summary

The mitochondrial membrane fatty acid profiles can be rapidly altered by dietary fat and omega-3 fatty acids administration, in particular, DHA can be effectively accumulated into mitochondria of both young and old mice. Also, DHA incorporation in plasma membrane has been observed. So, lipid profile change in mitochondrial membrane and plasma membrane both could impact respiratory complexes directly or indirectly respectively (Le Guen et al. 2015). DHA has been found to decrease total cholesterol content in the plasma membranes of DHA-treated cells which confers increasing plasma membrane fluidity of vascular EC (Hashimoto et al. 1999).

DHAs accumulate in cardiolipin (phospholipid), a component of inner mitochondrial membrane (Berger, Gershwin, and German 1992, Kitson et al. 2016, Khairallah et al. 2010, Ting, Chao, and Hsu 2015). Cardiolipin regulates various mitochondrial processes, including the electron transport chain and ATP synthesis (Pfeiffer et al. 2003, Acehan et al. 2011, Chen and Lesnefsky 2006, Chicco and Sparagna 2007, Paradies et al. 2011, Peyta et al. 2016). It has been shown that the content of cardiolipin is higher in glia cells compared to neurons (Adibhatla and Hatcher 2007, Kolomiytseva et al. 2010), which could attribute to the differential cell specific effects of DHA on mitochondrial biogenesis and activities after incorporating in mitochondrial membrane lipid-cardiolipin. Cardiolipin also minimizes the production of reactive oxygen species (ROS) and facilitates the transport of protons, which produce a strong electrochemical gradient across the inner mitochondrial membrane(Pointer and Klegeris 2017). Besides, as the result of its accumulation in cardiolipin, DHA is proposed to have amplified ROS production by modulating electron transport efficiency and increasing the production of superoxide radicals. It has previously been shown that cells treated with DHA die as the result of intracellular ROS production. These results suggest that cardiolipin acyl composition (containing DHA) can modulate the increased ROS production observed in aging and apoptosis (Watkins, Carter, and German 1998).

The MPTP is a large diameter (3 nm), high conductance, voltage-dependent channel that allows passage of water, ions, and molecules up to 1500 Da(Mnatsakanyan et al. 2017, Ricchelli, Šileikytė, and Bernardi 2011). Its opening causes mitochondrial depolarization and determines cell death under cell stress condition(Stanley, Khairallah, and Dabkowski 2012, Halestrap 2010). Ca^{2+}, oxidative stress and numerous reactive chemicals induce MPTP opening. Recently it was found that dietary supplementation with a mixture of DHA + EPA (70: 30 ratio) increase DHA and EPA in cardiac mitochondrial phospholipids and that increased tolerance of isolated mitochondrial to Ca^{2+}- induced MPTP opening(O'Shea et al. 2009, Stanley, Khairallah, and

Dabkowski 2012).In addition, DHA blocks the ER Ca^{2+} overload and depletion of Ca^{2+}_{ER} stores, as well as, the subsequent delayed rise in Ca^{2+}_{cyt} in astrocytes following *in vitro* ischemia which ultimately attenuates astrocyte death(Sergeeva, Strokin, and Reiser 2005).

Resolvin D1(RvD1) is the metabolite of DHA and it has been reported reducing mitochondrial swelling, lipid peroxidation and glutamate dehydrogenase release. Impaired activities of mitochondrial complexes I and III were restored by RvD1. RvD1 enhanced expression of the mitophagy-related proteins as well. It restored levels of mitochondrial biogenesis proteins including PPARγ coactivator 1α, nuclear respiratory factor 1 and mitochondrial transcription factor A and mtDNA level in disease model (Kang, Choi, and Lee 2018). There are no direct reports on DHA metabolism into resolvins or neuroprotectins by astrocytes. Use of the 5 and 15 LOXs potent inhibitor ebselen (enzymes involved in DHA to protectins and resolvins conversion) blocked the effect of DHA on ER Ca2+ loading and depletion that indicates involvement of DHA metabolites, possibly Neuroprotectin1 (NPD1) in astrocytes (Begum et al. 2012). Thus, this indirectly points towards DHA metabolism into its metabolites in astrocytes. The knowledge about DHA metabolism and elimination mechanisms in astrocytes will enhance our understanding about its therapeutic use. Or else, these metabolites could be investigated further to evaluate effects on mitochondrial respiratory chain complexes in MeCP2 deficient astrocytes. Interestingly, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a key regulator of enzymes that detoxify ROS, was induced by PDX (Protectin DX, a double lipoxygenase product from DHA) pre-treatment, suggesting a possible mechanism that explains the antioxidant effects of PDX in retinal pigment epithelium (RPE) cells (Dai et al. 2015).

In the current study, DHA normalized Uqcrc1 but did not alter Ndufv2 gene expression significantly. It also increased MeCP2 and BDNF genes expression in MeCP2 knock-down astrocytes. The western blot analysis showed increased MeCP2 and Ndufv2 proteins expressions in DHA treated MeCP2 knock-down astrocytes. In Spectrophotometric analysis of mitochondrial respiratory complexes also the enzyme activities were found to be increased in DHA treated MeCP2 knock-down astrocytes as compared to MeCP2 knock-down cells in a dose dependent manner. Also, the cytosolic calcium was restored in DHA treated MeCP2 knock-down cells. In spite of these alterations, ROS and mitochondrial membrane potential did not alter significantly in DHA treated MeCP2 knock-down astrocytes. In C6 glial cells, modulated intracellular calcium and ROS were observed in DHA treated MeCP2 knock-down cells.

Overall, the data indicates multiple target site and roles of DHA at micromolar (25,100μM) concentrations. When some parameters were rescued, the others were unaltered. This leads to no

conclusion for its use in MeCP2 deficiency involved diseases. DHA's effects in astrocytes are not as beneficial as reported in some other reports. Some other reports also advocate the opinion that DHA does not have health benefits (Sullivan et al. 2018). Further studies with more sensitive parameters might give us the clear clue on its therapeutic relevance with respect to mitochondrial functioning in astrocytes.