CHAPTER -5

Development of ex-vivo method for evaluation of mitochondrial modulators

5. Introduction

Mitochondrial toxicity is increasingly implicated as a contributing factor to many xenobiotic-induced organ toxicities. There is a need for predictive models or methods that can sensitively detect mitochondrial toxicity of chemical entities and their pharmacological effect in the early stage of the research and development process. A growing body of evidence suggests that defects in mitochondrial metabolism, particularly of the electron transport chain (ETC), play an important role in a wide range of pathogenesis of many neurological diseases such as Alzheimer's disease (Yao et al., 2009), Parkinson's disease (Reeve et al., 2018), Huntington's disease (Kim et al., 2010), and cardiovascular diseases (Higuchi et al., 1998). A recent study suggested that complex-1 inhibitors shifted the metabolism from oxidative phosphorylation (OXPHOS) to glycolysis, resulting in the production of lactate in cancer cells, which helps in the treatment of cancer (Yoshida et al., 2021). There are few models available that induce Parkinson's condition by inhibiting mitochondrial enzyme complex-1, such as rotenone (Heikkila et al., 1985; Ramsay et al., 1986; Krueger et al., 1990) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Nicklas et al., 1985; Ramsay et al., 1986), which are widely used model for Parkinson's disease by inhibiting mitochondrial function through complex-1 activity. MPTP enters into the mitochondria to inhibit nicotinamide adenine dehydrogenase (NADH) within mitochondrial respiratory chain complex-1 (Nicklas et al., 1985); (Ramsay et al., 1986), thus binding to the same site as rotenone, which is a classic

complex-1 inhibitor (Heikkila et al., 1985; Ramsay et al., 1986; Krueger et al., 1990). Impairment of mitochondrial respiration by MPTP is believed to decrease the levels of oxidized nicotinamide adenine dinucleotide (NAD) (Sonsalla et al., 1992).

However, these models are available and are limited to neurological diseases. Though rotenone and MPTP inhibit the complex-1 activity, they have also affected the other cell mechanism and irreversibly inhibit the complex-1 enzyme. Metformin is a pharmacological agent and specific complex-1 inhibitor and is distinct from the classical complex-1 inhibitor, rotenone and MPTP (Wheaton et al., 2014). Metformin requires a robust mitochondrial membrane potential to accumulate in the mitochondrial matrix and reversibly inhibits complex-1. By contrast, rotenone and MPTP are irreversible inhibitors of complex-1 that do not require mitochondrial membrane potential (Wheaton et al., 2014). Rotenone accumulation is not dependent on specific transporters expressed in the plasma membrane and readily accumulates in all cells and hence is highly toxic (Shaikh and Nicholson, 2009). Furthermore, metformin does not promote the generation of reactive oxygen species (ROS) from complex-1, while rotenone and MPTP stimulate ROS production from complex-1 (Wheaton et al., 2014). The positive charge of metformin has been proposed to account for its accumulation within the matrix of mitochondria that exhibit a robust inner mitochondria membrane potential. Hence, MET is a specific complex-1 inhibitor and is well tolerated *in-vivo.* We sought to determine that metformin might be a good candidate to develop the model to study mitochondrial function by inhibiting complex-1. Coenzyme-Q10 (Q10) and Resveratrol (RSV) are well-known complex-1 enzyme activators (Matthews et al., 1998; Desquiret-Dumas et al., 2013). Complex-1 plays a crucial role in maintaining mitochondrial homeostasis, not only

through its role in the energy metabolism and ROS production (Mourier & Larsson, 2011) but also by regulating the NAD+/NADH ratio (Santidrian et al., 2013). Therefore, Q10 and RSV have been chosen in this study as a positive control to compare the effect on normal and compromised mitochondrial function. Further, no such model is available to study, particularly the pharmacological or adverse effect of xenobiotics on mitochondrial function. There is a dire need to develop effective methods and therapeutics with tremendous potential for increasing and maintaining mitochondrial function. A standardized system to screen pharmacological agents that improve mitochondrial function would greatly facilitate this process. Our overall goal was to develop an easy and robust method for studying the effect of exogenous agents on mitochondrial bioenergetics. The *ex-vivo* approach developed enables to study the effects of pharmaceutical agents on the electron transport chain (ETC) function by adding different complex substrates and inhibitors to target the OXPHOS pathway. The efficiency of OXPHOS can be determined by adding a known amount of adenine-diphosphate (ADP) to isolated mitochondria and determining how much substrate is oxidized. Substrate oxidation was determined using a Clark oxygen electrode (Oxytherm) to measure oxygen consumption. The Oxytherm measures the current generated following reduction of oxygen. The current is proportional to the concentration of oxygen in the solution. Respiratory control rate (RCR) is the rate of oxygen consumption while ADP is being phosphorylated divided by the rate after the ADP is used up (Morton et al., 1996). Herein, we described the development and validation of a novel *ex-vivo* method that uses mitochondria isolated from mouse hepatic and pre-frontal cortex (PFC) brain tissue. Mitochondrial dysfunction was stimulated by treatment with metformin, and pharmacologic rescue was tested using

Q10 and RSV. In particular, the *ex vivo* system developed enables the testing of agents for their potential to cause mitochondrial dysfunction. This system also allows the testing for the ability of pharmacologic agents to improve mitochondrial respiratory function.

5.1. Materials and methods

5.1.1. Animals

All the experimental protocols were carried out following the principles of laboratory animal care (The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)) guidelines and approved by the Institutional Animal Ethical Committee (IAEC), Banaras Hindu University (Approval No. Dean/2019/IAEC/ 1254). Swiss albino adult male mice (25–30 g, eight weeks old; N = 15) were procured from the central animal house, Institute of Medical Sciences, Banaras Hindu University (IMS-BHU). The animals were kept in standard environmental conditions (25 ± 1 ◦C; relative humidity 55 ± 5%; and 12:12 h light/dark cycle), and all experiments were performed between 9:00 h and 18:00 h. Mice were housed in polypropylene cages (410 × 282 × 153 mm) (n = 6/cage) and provided food and water *ad libitum*. The animals were acclimated to housing for 15 days before the commencement of the experiments.

5.1.2. Chemicals

Resveratrol was received as a gift sample from Sami labs Bengaluru, Karnataka, India. Dimethyl sulfoxide (cat no. 67–68-5), metformin (cat no. 1115-70-4), and Coenzyme Q10 (cat no. 303–98-0) were purchased from Sigma-Aldrich, USA.

Mannitol (cat no. A648975), Sucrose (cat no. 27580), and bovine serum albumin (cat no. 97350) were procured from Himedia Pvt. Ltd. India. Potassium phosphate monobasic anhydrous (cat no. 7778-77-0), magnesium chloride (cat no. 7791-18-6), malate (cat no. 7554-12-3), pyruvate (cat no. 113–24-6), adenosine diphosphate (cat no. 119340–53-3), succinate (cat no 159389–75-0), oligomycin (cat no. 04876), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (cat no. 370–86-5), and rotenone (cat no. 83–79-4) were obtained from Sigma-Aldrich. Ethylene glycol-bis (β-aminoethyl ether)-N, N, N′ , N′ - tetra acetic acid (cat no. 62858) and 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (cat no. 16826) potassium salt was acquired from Himedia Pvt. Ltd.

5.1.3. Experimental design

Mitochondria from the hepatic and pre-frontal cortex tissue were isolated, and mitochondrial protein was determined. DMSO was used to dissolve the drugs; in the first experiment, safe concentration of DMSO for isolated mitochondria was assessed by incubating 100 μg mitochondria with varying concentrations of DMSO. In second experiment, the effect of MET (0.5 mg/ml), Q10 (0.5 mg/ml) and RSV (0.5 mg/ml) was determined. Then the concentration-dependent effect of Q10 (0.5, 1, and 2 mg/ml) was tested. Incubation was done using 100 μg mitochondria with DMSO and with different drugs for 10 min at 4 ◦C in each experiment. Mitochondrial bioenergetics and complex enzyme activity were determined.

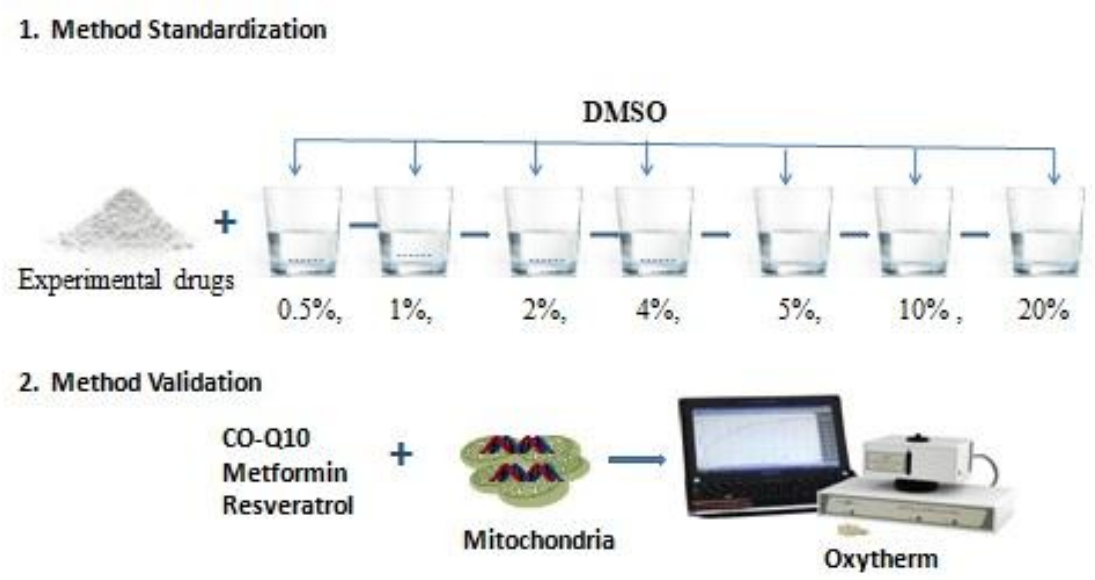

Figure 5.1 Study design

5.1.4. Determination of optimal DMSO concentration

DMSO is used as a diluents of Q10, RSV, and MET, but at high concentrations, it is known to be cytotoxic (Yuan et al., 2014). Testing of drug solubility and DMSO toxicity concentration was done using mitochondria isolated from the right lobe of the liver. Isolated mitochondria (100 µg) were incubated with 0.5%, 1%, 2%, 4%, 5%, 7%, 9%, 10% and 20% *v*/v DMSO. From this experiment, 5% DMSO was chosen as a solvent to dissolve the experimental drug, and thus all three drugs (Q10, RSV, and MET) were prepared in 5% DMSO for the experiments. *2.5. Preparation of drug, respiratory substrate, and inhibitor Drugs:* Q10, MET (0.5 mg/ml), and RSV (0.5 mg/ml) were dissolved in 5% DMSO. Q10 was prepared in varying concentrations of 0.5 mg/ml, 1 mg/ml, and 2 mg/ml to test the concentration-dependent effect on MET induce mitochondrial dysfunction. *Substrates:* 5 mM pyruvate, 2.5 mM malate, 200 mM ADP, and 10 mM succinate were freshly prepared in respiration buffer (125 mM KCl, 0.1% BSA, 20 mM HEPES, 2 mM MgCl2, 2.5

mM KH2PO4, pH 7.2). *Inhibitors:* 2 µM oligomycin, 2 µM FCCP, 1 µM rotenone were prepared in 100% ethanol and stored at-20 ◦C until further assays.

5.1.5 Isolation of mitochondria from the liver and brain

Isolation of mitochondria from the liver and pre-frontal cortex was done by the differential centrifugal method (Berman & Hastings, 1999) with some slight modifications (Samaiya & Krishnamurthy, 2015). Briefly, the right lobe of the liver and pre-frontal cortex (PFC) of the brain was dissected from mice, and 500 mg of tissue were homogenized with 20 strokes for the liver and 8 to 10 strokes for the brain in isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1%*w*/*v* bovine serum albumin, 20 mM HEPES buffer and 1 mM of EGTA in 100 ml of distilled water and pH adjusted 7.2 with KOH) using a glass Teflon tissue homogenizer (Thomas Scientific; USA). The homogenate was centrifuged at 1300 X g for 5 min at 4 ◦C. The supernatant was transferred to another tube and centrifuged at 14,000 X g for 10 min at 4 ◦C to pellet mitochondria. The supernatant was discarded, and mitochondria were washed by resuspending the pellets in an isolation buffer without EGTA and centrifuging at 14,000 X g for 10 min. Mitochondria were resuspended in 1 ml of respiration buffer (Samaiya & Krishnamurthy, 2015) and protein concentration was determined using the Lowry assay (Lowry et al., 1951) on a microplate reader (Biotek; Gen5, USA).

5.1.6 *Ex-vivo* incubation and measurement of mitochondrial respiration

Mitochondrial suspensions of 100 µg in 20 µl of respiratory buffer were prepared, and experimental drugs were added at varying concentrations and incubated for 10 min at 4 ◦C. Mitochondrial function was assessed using an Oxytherm Clark-

type oxygen electrode (OXYT1/ED, Hansatech Instruments, Norfolk, UK) with the standard setting to measure oxygen in nmol/ml. Briefly, mitochondria (100 μg/20 μl; presence or absence of pharmacologic agent) were placed in a sealed Oxytherm chamber containing 500 μl respiration buffer and continuously stirred at 37 ◦C, with assays set to run over 10 min. Substrate for complex-1 pyruvate/malate (P/M) was added after 2 min following addition of mitochondrial suspension to the Oxytherm chamber, ADP, Oligomycin, FCCP, Rotenone, and succinate were added sequentially at 1 min intervals. The oxygen consumption rate was defined as the slope of the response of isolated mitochondria to the consecutive administrations of each substrate (Gilmer et al., 2010). In this system, State II was initiated by the addition mixture of 5 mM pyruvate and 2.5 mM malate (P/M substrate) as substrates for Complex-1 of the *ETC.* State III respiration was initiated by activation of ATP synthase (Complex-4) *via* the addition of ADP (200 mM). State IV occurs when ATP synthase is inhibited by the addition of oligomycin (2 μM). The addition of FCCP (2 μM) induces State V by uncoupling electron transport from oxidative phosphorylation. Rotenone (1 μM) was added to inhibit complex-1 activity, followed by succinate (10 mM) to measure respiration through Complex-2, termed as state-VI. The respiratory control ratio (RCR) was reported and calculated as the ratio of state III/state IV oxygen consumption. In addition, the different complex enzyme activity was assessed by colorimetric and fluorometric methods to validate the method.

5.1.7. Mitochondrial ETC enzyme activities

5.1.7.1. NADH dehydrogenase (Complex-1) activity

The oxidation of NADH in the presence of potassium ferricyanide, which is an artificial electron acceptor, was assayed fluorometrically (Shapiro et al., 1979). As per

the protocol, the nonenzymatic reaction was measured by the addition of 100 µl of mitochondrial suspension to the reaction mixture, which consisted of 200 µl of 10 mM potassium ferricyanide, 60 µl of 1 mM NADH in 2 mM potassium phosphate buffer, and 2.64 ml of 0.12 M potassium phosphate buffer. (PH 8.5; incubated at room temperature for 5 min) The reaction was assayed by florescent microplate reader, using 350 nm excitation and 470 nm emission wavelengths. The complex-1 activity was expressed as nmol NADH oxidized/min/mg protein.

5.1.7.2 Succinate dehydrogenase (Complex-2) activity

Succinate dehydrogenase (SDH) activity in the mitochondrial fraction was estimated by the spectrophotometric method as previously described with minor modification (Old & Johnson, 1989). The method involves oxidation of succinate to fumarate by nitro blue tetrazolium (NBT), an insoluble colored compound and reaction indicator. It is an artificial electron acceptor which itself reduces and makes diformazan. Succinate to fumarate conversion leads to the release of H+ ions which mediated the reaction of NBT. The reaction mixture consisted of 0.1 M phosphate buffer (0.2 M NaH2PO4 and 0.2 M Na2HPO4); pH 7.0, 130 mM sodium succinate, 1.5 mM NBT, 0.2 mM phenazine methane sulphate (PMS) and 1 mM sodium azide. Mitochondria (100 µg in 30 µl of respiration buffer with 5% (*v*/v) DMSO) were added to 50 µl of the reaction mixture and incubated for 30 min. Ethyl acetate 500 µl (99%) was added, and the mixture was agitated. After phase separation, the organic ethyl acetate fraction was extracted, and the absorbance of this phase was measured at 570 nm on a microplate reader to determine the concentration of NBT-diformazan produced. The complex-2 activity was reported as micromole formazan produced/min/mg protein.

5.1.7.3. Cytochrome-C oxidase (Complex-4) activity

The complex-4 activity was measured in mitochondrial preparation according to (Storrie & Amadden, 1990). At first, cytochrome-C was reduced by adding a few crystals of sodium borohydride, which was later neutralized to pH 7.0 with 0.1 M HCl. The reduced cytochrome-C (0.3 mM) was added to 0.075 M phosphate buffer (pH 7.4), and 100 µl mitochondrial suspension was added to initiate the reaction. The reduction in absorbance was measured at 550 nm for 3 min, and the results were expressed as nmol cytochrome-C oxidized/ min/mg protein (ε550 = 19.6 mmol-1 cm-1).

5.1.7.4. F1F0 ATP synthase (Complex-5) activity

The complex-5 activity was measured following the method as previously described (Griffiths & Houghton, 1974). Briefly, 500 ml of ATPase buffer (50 mM Tris-HCl and 5 mM MgCl2, pH 7.5) with 5 mM ATP was used to incubate mitochondrial suspension at 37 ◦C for 10 min. After the addition of 500 ml of 10% (*w*/*v*) trichloroacetic acid, the reaction was stopped. The contents were centrifuged at 3000 *g* for 20 min, followed by mixing 500 ml of supernatant with an equal amount of water. After that, the inorganic phosphate concentration was measured by the earlier described methods (Fiske & Subbarow, 1925), and the results were expressed as nmol ATP hydrolyzed/min/mg protein.

5.1.7.5 Mitochondrial Membrane Potential (MMP) activity

Tetra-methyl rhodamine methyl ester (TMRM), a fluorescent cationic dye, is used to monitor mitochondrial membrane potential. Initially, 100 µl of the

mitochondrial suspension was added to 890 µl of assay buffer (80 mM NaCl, 75 mM KCl, 25 mM D-glucose, and 25 mM HEPES, pH 7.4), followed by the addition of 10 µl of 15 µM of TMRM solution to the above mixture and incubated for 15 min at 37 ◦C. After incubation, 400 µl of PBS was added and centrifuged at 10,000 X g. resulting, pellets were collected in PBS. The rhodamine dye taken up by mitochondria was measured with a Biotech instrument, USA (Huang, 2002). The fluorescence emission was read at an excitation λ of 535 ± 10 nm and emission λ of 580 ± 10 nm using slit no. 10. The peak fluorescence intensity was recorded at 570 ± 5 nm, and results were expressed as fluorescence intensity/mg protein.

5.1.8. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Data were analyzed using one-way ANOVA followed by Tukey *post hoc* test. Statistical analyses were performed using the GraphPad Prism software (GraphPad Software Inc., San Diego, USA) version 8.0.2. All the data analyses $p < 0.05$ was considered to be statistically significant.

5.2. Results

5.2.1. DMSO was toxic at >5% to isolated mitochondria

Titration assays were performed using 0.5%, 1%, 2%, 4%, 5%, 7%, 9%, 10% and 20% DMSO to determine the solubility of Q10, RSV and MET, and to measure the effect of DMSO on mitochondrial respiration rates. Below 5% DMSO, Q10 was not solubilized, and above 10% concentration, respiration of mitochondria isolated from the liver was inhibited (as shown in Fig. 5.2 (a) and (b)). In the presence of 5%

DMSO, respiration of mitochondria was not affected. Therefore, for all the remaining assays, 5% DMSO was used to dissolve the drugs and as a comparator.

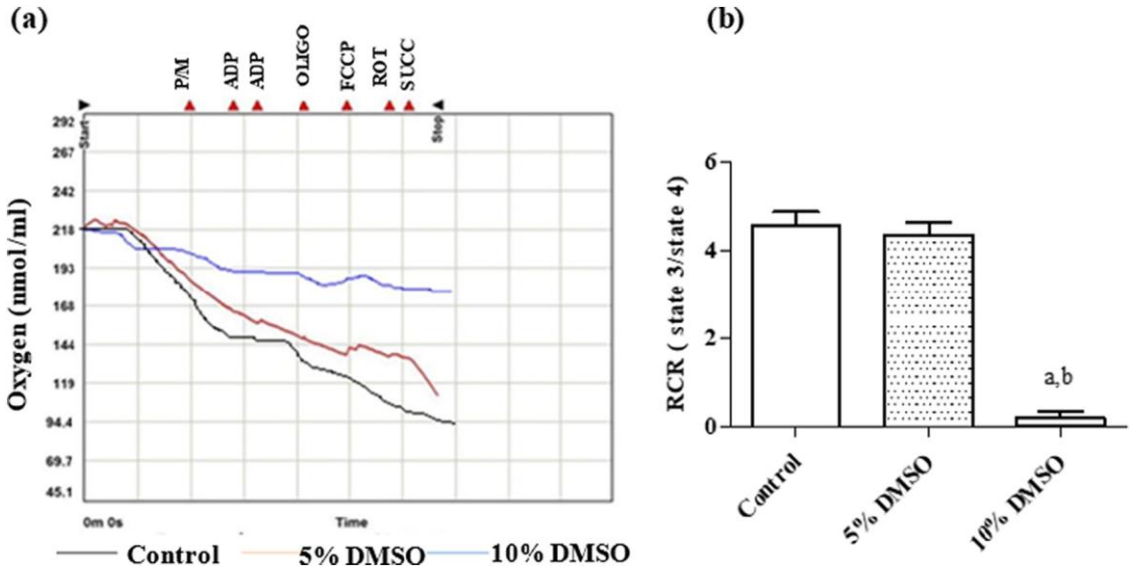

Figure. 5.2 (a) The effect of 5% and 10% DMSO diluents on mitochondrial bioenergetics (Oxygraph). Baseline = State-I, P/M; State-II, ADP; State-III, OLIGO (Oligomycin); State-IV, FCCP; State-V, ROT (Rotenone); Inhibitor for complex-1, SUCC (Succinate); State-VI. (b) Respiratory control ratio (RCR). Values are RCR in mean ± SD (n = 3) $^{a}p < 0.05$ compared to control, $^{b}p < 0.05$ compared to 5% DMSO. One-way ANOVA followed by Tukey's *post hoc* test.

5.2.2. MET inhibited mitochondrial respiration; Q10 improved MET inhibited mitochondrial respiration

When Q10 (0.5 mg/ml) was added to the isolated liver mitochondria, RCR increased 1.5-fold relative to control and 5% DMSO vehicle-treated mitochondria (Fig. 5.3a). The addition of MET (0.5 mg/ml) decreased RCR to <95% of control levels. When Q10 was added to MET pretreated mitochondria, the RCR ratio was recovered i.e., increasing to about 82% of the rate of control. The response of mitochondria isolated from the pre-frontal cortex (PFC; Fig. 5.3b) was similar to the effects of Q10 and MET treatment on liver mitochondrial RCR. Relative to control,

Q10 increased RCR 1.5-fold in brain mitochondria *ex-vivo*. MET decreased RCR to <88% of controls. When Q10 was added in the presence of MET, RCR recovered some to 76% of control. There was also a 1.5-fold increase in RCR when RSV 0.5 mg/ml was added in the last step to validate the method.

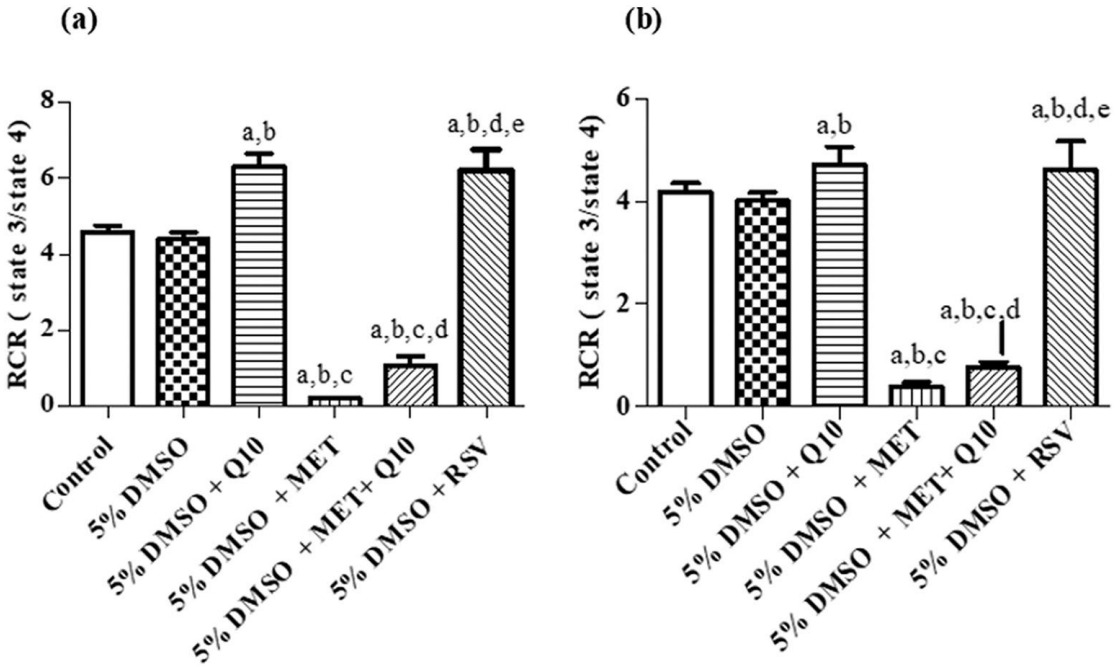

Figure. 5.3 (a) The effect of Q10, RSV and MET on respiratory control ratio (RCR) of isolated liver and (b) brain mitochondria. Data in mean ± SD (n = 3). $^{a}p < 0.05$ compared to control, $^{b}p < 0.05$ compared to 5% DMSO, $^{c}p < 0.05$ compared to 5% DMSO + Q10, $^{d}p < 0.05$ compared to 5% DMSO + MET and $^{e}p < 0.05$ compared to 5% DMSO + Q10 + MET. One-way ANOVA followed by Tukeys *post hoc* test.

5.2.3. Q10 exerts a concentration-dependent effect on MET induced mitochondrial respiration

The ability of varying concentrations of Q10 (0.5 mg, 1 mg, and 2 mg/ml) was tested on normal function and MET-induced mitochondrial dysfunction in the brain. All Q10 concentrations tested improved the RCR to the same extent, suggesting that maximal rate was achieved in the system. However, a concentration-dependent effect of Q10 was found for its ability to restore RCR following MET-induced dysfunctional

mitochondria (Fig. 3). The addition of 0.5 mg/ml Q10 only increased RCR modestly of control, however, 1 mg/ml increased it to 98% of control, whereas 2 mg/ml restored and improved RCR to 100% of control levels. The addition of RSV to MET-induced dysfunctional mitochondria also improved their function to the same extent.

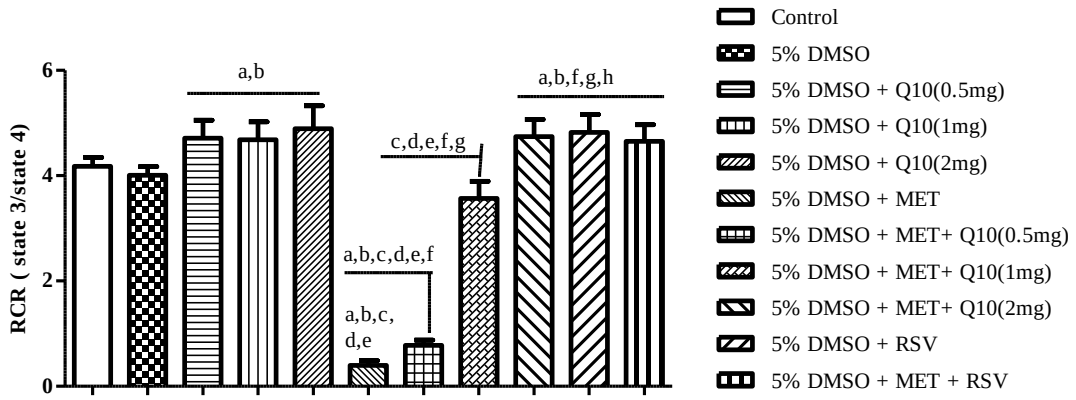

Figure. 5.4 Respiratory control ratio (RCR) in mitochondria isolated from the PFC of the mouse brain. Values are in mean ± SD (n = 3). $^{a}p < 0.05$ compared to control, $^{b}p < 0.05$ compared to 5% DMSO, $^{c}p < 0.05$ compared to 5% DMSO + Q10 (0.5 mg), $^{d}p < 0.05$ compared to 5% DMSO + Q10(1 mg), $^{e}p < 0.05$ compared to 5% DMSO + Q10 (2 mg), $^{f}p < 0.05$ compared to 5% DMSO + MET, $^{g}p < 0.05$ compared to 5% DMSO + MET + Q10 (0.5 mg) and $^{h}p < 0.05$ compared to 5% DMSO + MET + Q10(1 mg). One-way ANOVA followed by Tukeys *post hoc* test.

5.2.4. Q10 exerts concentration-dependent effect on MET-induced mitochondrial dysfunction on complex-1, 5 with no effect on complex-2 and 4

To validate and verify the *ex vivo* approach, the activity of mitochondrial enzyme complexes 1, 2, 4, and 5 were measured with colorimetric and fluorometric assays in parallel with mitochondrial membrane potential. The addition of Q10 at all concentrations tested improved complex 1, 2, 4, and 5 activities to a similar degree over vehicle control (5% DMSO); (Fig. 5.5). There was no effect of MET treatment on complex-2 (Fig. 5.5b), and complex-4 (Fig. 5.5c) activities. A significant

concentration-dependent effect of Q10 on mitochondrial enzyme complex-1 (Fig. 5.5a) and complex-5 (Fig. 5.5d) activities were found for PFC mitochondria pretreated with MET to induce dysfunction. Thus, Q10 was found to improve complex-1 and 5 activities in a concentration-dependent manner against MET.

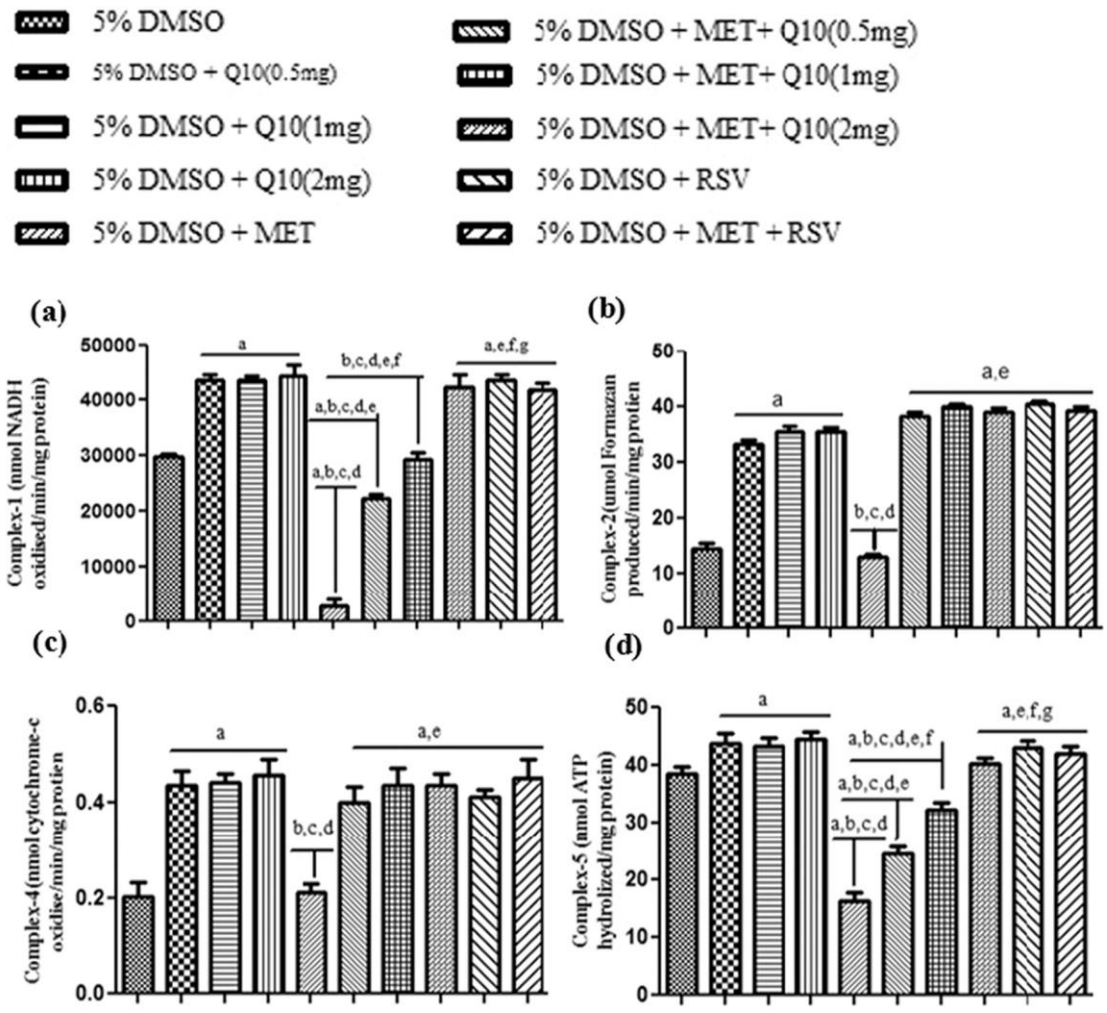

Figure. 5.5 Complex-1 (a), 2 (b), 4 (c) and 5 (d) activity in isolated brain mitochondria. Values are in mean ± SD (n = 3). $^{a}p < 0.05$ compared to 5% DMSO, $^{b}p < 0.05$ compared to 5% DMSO + Q10 (0.5 mg), $^{c}p < 0.05$ compared to 5% DMSO + Q10 (1 mg), $^{d}p < 0.05$ compared to 5% DMSO + Q10 (2 mg), $^{e}p < 0.05$ compared to 5% DMSO + MET, $^{f}p < 0.05$ compared to 5% DMSO + MET + Q10 (0.5 mg) and $^{g}p < 0.05$ compared to 5% DMSO + MET + Q10(1 mg). One-way ANOVA followed by followed by Tukeys *post hoc* test.

5.2.5. Q10 improved MET-induced changes in mitochondrial membrane potential (MMP)

The addition of Q10 at all concentrations tested, increased the MMP (Fig. 5.6) relative to vehicle control (5% DMSO) in PFC. MET significantly decreased the membrane potential to 60% of control. Membrane potential was rescued in MET pretreated mitochondria of the PFC in a concentration-dependent manner with Q10 and RSV.

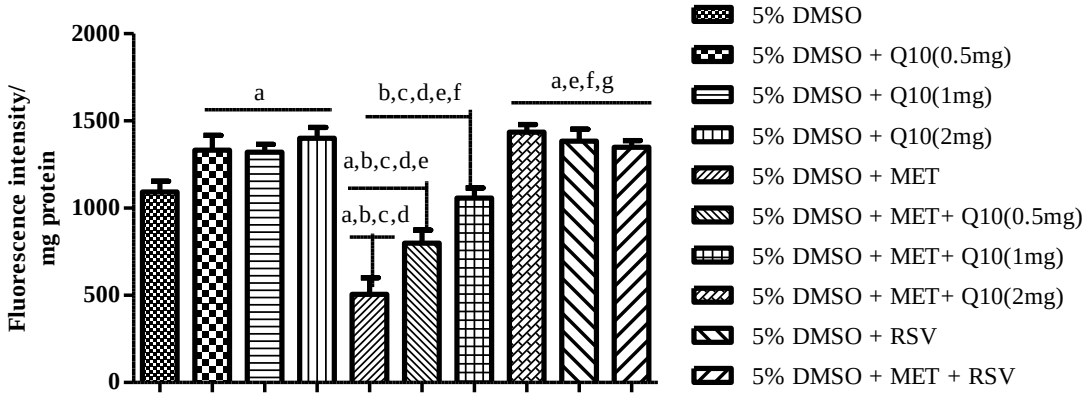

Figure. 5.6. Mitochondrial membrane potential (MMP) in isolated brain mitochondria. Values are in mean ± SD (n = 3). $^{a}p < 0.05$ compared to 5% DMSO, $^{b}p < 0.05$ compared to 5% DMSO + Q10 (0.5 mg), $^{c}p < 0.05$ compared to 5% DMSO + Q10 (1 mg), $^{d}p < 0.05$ compared to 5% DMSO + Q10 (2 mg), $^{e}p < 0.05$ compared to 5% DMSO + MET, $^{f}p < 0.05$ compared to 5% DMSO + MET + Q10 (0.5 mg) and $^{g}p < 0.05$ compared to 5% DMSO + MET+ Q10 (1 mg). One-way ANOVA followed by Tukeys *post hoc* test.

5.3 Discussion

The overall aim of the study was to develop and validate an *ex-vivo* system that can be used for medium to high throughput screening of potential therapeutics that affect mitochondrial bioenergetics at the early stages of drug discovery. We describe a novel, robust, and economical method for *ex-vivo* testing of drugs on

mitochondrial bioenergetics in normal and dysfunctional states and further verified by using resveratrol. This method was validated by measuring the effect of all three drugs on the different complex enzymes of ETC, and the results were found to correlate to mitochondrial bioenergetics. Hence, this method can be used to test the adverse and toxic effects of new drugs on mitochondrial function that can be compared to the effects of MET. Moreover, the potential of the therapeutic rescue of a drug can be compared by adding to MET pretreated mitochondria. Thus, the validated *ex-vivo* method provides a system to study the efficacy of potential therapeutics on mitochondrial dysfunction and rescue. Since many drugs are lipophilic, suitable diluents must be used. In the laboratory, DMSO is commonly used to solubilize drugs. Here we showed that DMSO could be used for up to 5%. Below 5%, Q10 was not solubilized; however, above 5%, it inhibited mitochondrial respiration. Secondly, the *ex-vivo* system was developed using mitochondria isolated from the liver and the PFC of the brain. These tissues were selected as they are the primary target tissues for drug development and toxicity testing. Mitochondrial dysfunction underlies the pathogenesis of several neurologic diseases, including Alzheimer's (Yao et al., 2009) and Parkinson's disease (Reeve et al., 2018). Moreover, following absorption across the gastrointestinal tract, the liver is often the first site where a drug is metabolized *in vivo* (Kulsharova and Kurmangaliyeva., 2021).

At high concentrations, mitochondria in this organ are often most vulnerable to toxic side-effects of drugs (Labbe et al., 2008). The effects of the three drugs tested, RSV, Q10, and MET, were similar across both hepatic and PFC mitochondria. MET was used to induce mitochondrial dysfunction and we found that MET treatment completely inhibited the consumption of oxygen, as indicated by the RCR, the ratio of

state III/state IV oxygen consumption. These findings were consistent with studies that showed mitochondria treated with MET reduced RCR through the electron transport chain (Liu et al., 2014). In addition to showing an overall decrease in mitochondrial respiration, there was a shift in favor of uncoupling of ETC. As a result, mitochondrial metabolism becomes energetically inefficient, and cells compensate for this limitation in ATP production by increasing aerobic glycolysis (Andrzejewski et al., 2014). This is consistent with the evidence for an inhibitory effect on complex-1 together with a membrane potential-driven accumulation of positively charged drugs within the mitochondrial matrix (Owen, et al., 2000). Validating the studies, we found that not only did MET decrease mitochondrial complex-1 activity, it also affected complex-5 activity and membrane potential, but did not affect activities of complex-2 and 4. Q10 at 0.5 mg/ml, 1 mg/ml, and 2 mg/ml increased RCR to basal level in normal mitochondria, suggesting the maximal rate of oxygen consumption had been met. However, there was a concentration-dependent effect of Q10 on the rescue of MET-induced mitochondrial dysfunction, with 2 mg/ml concentration completely restoring RCR to control levels. The improvement in RCR is likely due to the combined effects of Q10 on mitochondrial complex enzyme activities and membrane integrity, as activities of individual enzymes were increased in all complexes tested (1, 2, 4, and 5), as well as the membrane potential. Q10 has directly improved the mitochondrial ATPase activity by supplying additional electrons from complex-2 to complex-4 and ultimately increased the ATP synthesis through the enzyme complex system. Q10 rescue of MET-induced mitochondrial dysfunction was found to be concentration-dependent for complex-1, 5, and membrane potential. This suggests that Q10 may have competed with MET for same

sites on these enzyme complexes. Further, drug-induces mitochondrial dysfunstion can lead to development of potential side effects. Metformin inhibits complex-1 (NADH ubiquinone oxidoreductase I) of the electron transport chain of the oxidative-reduction pathway that mediates the transfer of protons across the mitochondrial membrane. Therefore, a standardized system for evaluating the effects of exogenous agents on mitochondrial function could also be used to screen for potential drug toxicity. Several other complex-1 inhibitors, such as insecticides and acaricides, can also be used in place of MET to simulate the condition based on their mechanism of action by inhibiting complex-1 enzyme activity in ETC (Lümmen., 1998). Rotenone and piericidin A were known for long as high-affinity inhibitors of proton-translocating NADH ubiquinone oxidoreductase (Lümmen., 1998). In enzymatic assays with membrane-bound or solubilized NADH ubiquinone oxidoreductase, the complex-1 active insecticides closely resembled the classical inhibitors rotenone and piericidin which block electron transport between iron-sulfur cluster and ubiquinone. Resveratrol is a mitochondrial drug that is known to act on complex-1 and improve its function (Desquiret-Dumas et al., 2013). RSV was used as a standard reference drug in the method development to compare the effect of Q10 and added in the last step of the experiment. RSV improved RCR and different complex enzyme activity-independent to MET effect and confirmed that the compromised result of mitochondria is due to MET. Further research is needed to compare the *ex-vivo* study with *the in-vivo* analysis to validate its potential for high throughput screening of drugs. This method is simple, repeatable, robust, and economical at the laboratory level.

5.4 Summary

MET directly acts on mitochondria and inhibits the complex-1 enzyme in the OXPHOS system. MET-mediated decrease in mitochondrial function and integrity was reversed by a Q10 compensatory increase in function and integrity. Hence, this *ex-vivo* method can be used to assess the effect of several drugs acting on the mitochondria. These drugs could be potentially used in pathophysiological conditions associated with metabolic disturbances by improving the mitochondrial function and their integrity. Further, we could use these compounds to minimize the mitochondrial toxicity of drugs such as MET.