Ca^{2+} INDUCED MITOCHONDRIAL MEMBRANE POTENTIAL DISSIPATION ESCALATES OXIDATIVE STRESS IN AGED RATS

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Abstract. The mitochondrion is the focal point of bioenergetic interactions that manage various cell functions like Ca^{2+} flux management, programmed cell death, reactive oxygen species (ROS) generation, and energy dynamics involving ATP generation and dissemination to the cell. The current study conducted to understand the role of Ca^{2+} in varying mitochondrial membrane potentials in alleviating the oxidative burden on the cell. Brain mitochondria, isolated from rats aged 2–3 weeks (neonatal), 2–3 months (young adult), and 2–3 years (aged) of age, were used in the study. Mitochondria isolated from aged rats displayed elevated ROS production levels upon Ca^{2+} treatment, measured as a function of DCF fluorescence intensity. Rhodamine-123 fluorescence measured the alterations in mitochondrial membrane potential, indicating a significant depolarization in the mitochondria isolated from aged rats as compared to mitochondria isolated from the other age groups. Experiments with ruthenium red, an effective blocker of Ca^{2+} uniporter, confirms the Ca^{2+}-dependent membrane depolarization and ROS generation. Large mitochondrial membrane potential dissipation observed in the aged rats explains the vulnerability of the same to oxidative stress during aging.

Key words: Calcium, membrane potential, reactive oxygen species, aging, mitochondria.

INTRODUCTION

An undeniable fact of life is an incomprehensible death, set off by a series of biochemical and physiological functional flaws. However, the underlying scientific reasoning on aging is short by an acceptable explanation. One mechanism that has prevailed widely in convincing researchers is the age-associated degeneration triggered by the release of reactive oxygen species (ROS). ROS is generated intracellularly at various locations by numerous enzymes participating in a biochemical reaction. Plasma membrane-bound redox proteins, such as the emerging family of xanthine oxidases, nitric oxide synthases, and NADPH oxidases [19] peroxisome contained both α and β oxidation of fatty acid, and lipid metabolism [29] as well as innumerable cytoplasmic enzymes [21]. Although all

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these sources majorly contribute to the vast expanse of intracellular oxidative load, cellular ROS (estimated at approximately 80–90 %) can be extensively mapped back to the mitochondria [8]. Reactive oxygen species generated by the mitochondrial respiratory chain complex enzymes causes oxidative damage to mitochondrial DNA (mtDNA) [14], protein and lipid, and subsequent decline in mitochondrial activity. Apart from its primary role of synthesizing ATP [4], Ca^{2+} buffering [13] and apoptosis [23], mitochondria are also involved in the generation of a copious amount of highly reactive free radicals [25].

In aging and neurodegenerative disorders, Ca^{2+} is improperly regulated when neurons are depolarized, allowing a massive influx of Ca^{2+} through glutamate receptors. Glutamate receptor implicated Ca^{2+} dys-homeostasis is the leading cause of neuronal cell death [6]. However, the downstream cellular events to blame for neuronal/cellular dysfunction are not clearly known. Studies on primary neuronal culture [9] and cortical slices [16] have suggested the involvement of mitochondria in the free radical generation, ensuing catastrophic neuronal death. Although differential Ca^{2+} entry through N-methyl-D-aspartate (NMDA) and non-NMDA receptors, leading to glutamate excitotoxicity [24], has been identified, mitochondrial calcium flux leading to elevated oxidative load, has been elusive; thus, leaving a lacuna to be addressed by a systematic study. The increased levels of mitochondrial Ca^{2+} pushes the cell to a self-induced suicide mission by cytochrome c release and ATP depletion [18], followed by elevated ROS discharge levels. These mechanisms have suggested the phenomenon of oxidative stress being the first overriding step in aging and neurodegeneration.

Our study focuses on identifying the role of calcium in inducing mitochondrial membrane potential dissipation and estimating the coupled ROS production. This study is done on isolated rat brain mitochondria from three different age groups. The study quantifies the calcium-induced oxidative burden in all the three age groups and extends to measure the altered mitochondrial membrane potential, asserting calcium's crucial position in oxidative stress.

MATERIALS AND METHODS

MATERIALS

Ethleneglycol-bis-(-aminoethyl ether) N, N, N1, N1 tetraacetic acid (EGTA), ruthenium red, calcium chloride, sodium succinate, N-2-hydroxyethylpiperazine-n-2-ethanesulfonic acid (HEPES), and dimethyl sulfoxide were obtained from Sigma Chemicals Co., 2'-7'-dichlorofluorescein diacetate (DCFH-DA) and Rhodamine-123 (Rh-123) were obtained from Molecular Probes, USA. Other chemicals of analytical grade were obtained from BDH Industries Ltd and Glaxo Laboratories, Mumbai, India.

Sprague dawley albino rats were procured from Sri Raghavendra Enterprises, Bangalore, and acclimatized to laboratory conditions (12 h dark/light, 28±2 °C). Animals were let free to feed on standard food (Amruth Feeds, India) and potable water *ad libitum*. Animals were maintained in accordance with the stipulated guidelines of ICMR − National Institute of Nutrition, Hyderabad, with the approval taken from Animal Ethical Committee, Bangalore University, Bangalore.

Rats of three different age groups: 2−3 weeks, 2−3 months and 2−3 years, were chosen to correlate neonatal, young adult and senescent/old stage of the animal. They were acclimatized a week before the commencement of the study.

METHODS

Isolation of mitochondria

Experiments on all animals were done in following the guidelines specified by ICMR − National Institute of Nutrition, India, and as per the approved protocol of the ethical committee.

Mitochondria were isolated from the whole brain of rats by conventional differential centrifugation, as described by [17] with minor modifications. After extraction, the brain tissue was placed in isolation media containing HEPES buffer (pH adjusted to 7.2 with KOH), 1 mM tetrapotassium EDTA, 75 mM sucrose, and 20 mM 0.1 % fatty acid-free bovine serum albumin (BSA). The tissue was minced and ground to form a homogenate, which was mixed with the buffer in 1:5 ratio. Following centrifugation at 4,000 rpm, the first pellet (nuclear fraction) was removed. The supernatant collected was layered over 1.2 M sucrose gradient and centrifuged at 20,000 rpm for 20 minutes. The mitochondria collected as pellets were treated with storage buffer similar to isolation buffer, except that it contained 0.1 mM EDTA and was without BSA. The solution was washed with storage buffer and spun at 10,000 rpm for 10 minutes. The mitochondria collected were stored at −40 °C. All the isolation procedures were carried out at 0−4 °C to retain maximal activity of the isolate. The total protein was concentrated to 1 mg/mL in storage buffer upon Lowry's method of estimation.

Mitochondrial marker assay

Succinate dehydrogenase (SDH), *aka* complex II of the electron transport chain, is a marker enzyme of mitochondria and entails checking the health of the sample isolated. The SDH assay uses succinate as a substrate and 2,6-dichlorophenolindophenol (DCPIP) as an electron acceptor. To effectively activate Complex II, the sample was freeze-thawed before using. All three age groups of SDH activities were measured for statistically significant results to indicate their viability (data not shown).

Measurement of mitochondrial membrane potential changes

Rhodamine 123 (Rh-123), a fluorescent dye, was used to monitor membrane potential alteration [1] in mitochondria isolated from rat brain. However, mitochondria, if energized, quench Rh-123 fluorescence. The variation in fluorescence intensity was directly correlative of altered mitochondrial membrane potential. Rh-123 being cationic localizes in the mitochondria, forming aggregates and dissociates upon depolarization to form monomers that result in increased fluorescence.

0.1 μg of Rh-123 is used to measure the potential membrane changes of 1 mg mitochondria. Before measurement, mitochondria were treated with Rh-123 for a minute in the fluorometric cuvette, provided with a slow stirring magnetic bead. Once the signal was stabilized, and membrane potential maintained, a 6-minute protocol was followed, which consisted of Ca^{2+} addition at the 120^{th} second and the measurement of the alteration in mitochondrial membrane potential. The fluorescent probe was excited at 501 nm, and fluorescence collected at 528 nm. Increased fluorescence indicated the depolarization of membrane potential.

Measurement of reactive oxygen species in mitochondria

To enable free radical measurement, the isolated mitochondria were incubated with 2',7'-dichlorofluorescein diacetate (DCFH-DA) in a cuvette provided with a magnetic bead for slow stirring. The probe diffused into the mitochondria, and the mitochondrial esterases hydrolyzed the acetate groups, leading to DCHF, which reacted with the oxidants generated, resulting in the observed fluorescence [10]. The method resulted in the proper loading of dye with high fluorescent intensity. DCFH-DA was prepared in DMSO and working stock solution in incubation buffer (75 mm sucrose, 20 mm HEPES, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, 0.1 % BSA) maintained at pH 7.3.

1.5 mL of cuvette solution included incubation buffer, 5 mM succinate, and 100 μg mitochondrial protein, along with 10 μM DCFH-DA. The fluorescent probe was excited at 492 nm, and fluorescence collected at 530 nm. The whole experiment lasted for 10 minutes, wherein 100 μg mitochondria were added at the 60^{th} second. At the 240^{th} second, the mitochondria were treated with 100 μM Ca^{2+}. An increase in fluorescence indicated the rise in a free radical generation.

Statistical analysis of data

The data has been represented as mean ± SD and the respective number of experiments performed is mentioned along with the figures. For tests of significance, the three-way analysis of variance and Bonferroni tests were performed, considering a $p < 0.001$ as significant.

RESULTS

Age-dependent changes in mitochondrial functions were studied by measuring the mitochondrial membrane potential alterations and formation of ROS in mitochondria isolated from the brain of *Sprague dawley* rats that were neonatal, young adult and aged. The study attempts to illuminate the link between Ca^{2+} and membrane potential variations and understand whether differential mitochondrial functions could be responsible for higher sensitivity to oxidative stress during aging.

Ca^{2+} DEPENDENT CHANGES IN MITOCHONDRIAL MEMBRANE POTENTIAL

Membrane potential changes in isolated mitochondria were monitored using Rh-123. The mitochondria treated with Rh-123 to varying concentration (10 µm, 50 µm, 100 µm, 200 µm, 500 µm and 1 mM) of Ca^{2+} showed the graded response of variation in membrane potential while their basal level fluorescence being maintained at a steady-state, indicating the inherent potential of an age group (Fig. 1).

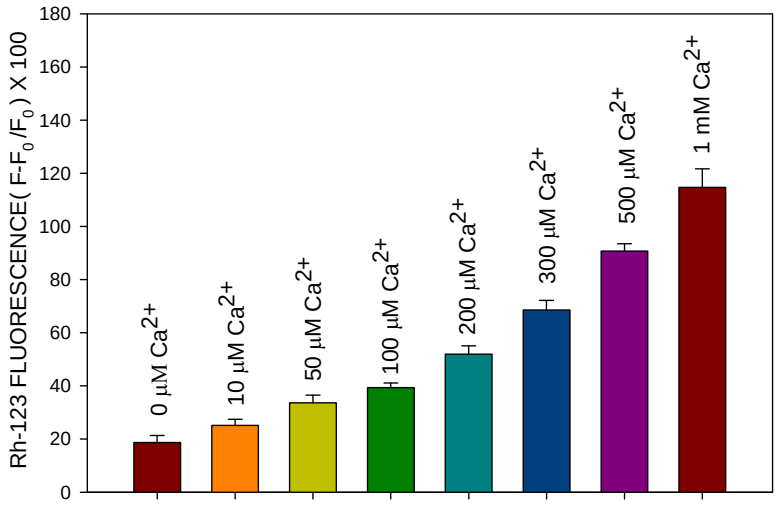

Fig. 1. Effect of Ca^{2+} on alteration in membrane depolarization in mitochondria. There was linear increase in the fluorescence with increased concentration of calcium. F_0 is the fluorescence intensity without Ca^{2+} and F is the fluorescence intensity measured with Ca^{2+}. Data points represent the mean ± SD of 6 experiments ($p < 0.001$).

The mitochondria isolated from aged rats showed 24.64 % higher depolarization as compared to neonatal rats. After the calcium challenge, the

mitochondria isolated from aged rats showed a higher degree of mitochondrial depolarization as compared to neonatal and young adult rats. There was a 258.8 % increase in mitochondrial depolarization observed in the mitochondria obtained from aged rats. However, the mitochondria isolated from neonatal and young adult rat's brain showed 170.1 % and 110.62 % rise as compared to their respective controls.

EFFECT OF RUTHENIUM RED ON Ca^{2+} INDUCED MITOCHONDRIAL MEMBRANE POTENTIAL CHANGES

Upon treatment with 2.5 µM ruthenium red followed by exposure to 100 µM Ca^{2+}, the mitochondria isolated from aged rats showed 45.79 % less depolarization as compared to the control-treated with calcium alone. Mitochondria isolated from neonatal and young adult rats showed 43.89 % and 6.04 % decrease in the dissipation of potential, respectively. However, in the presence of 5 µM ruthenium red and 100 µM of Ca^{2+}, the mitochondria isolated from aged rat brain showed a –29.32 % decrease in depolarization as compared to the control-treated with Ca^{2+} alone. Mitochondria isolated from neonatal and young adult groups showed –3.75 % and –17.8 % less alteration in membrane potential respectively, as shown in (Fig. 2).

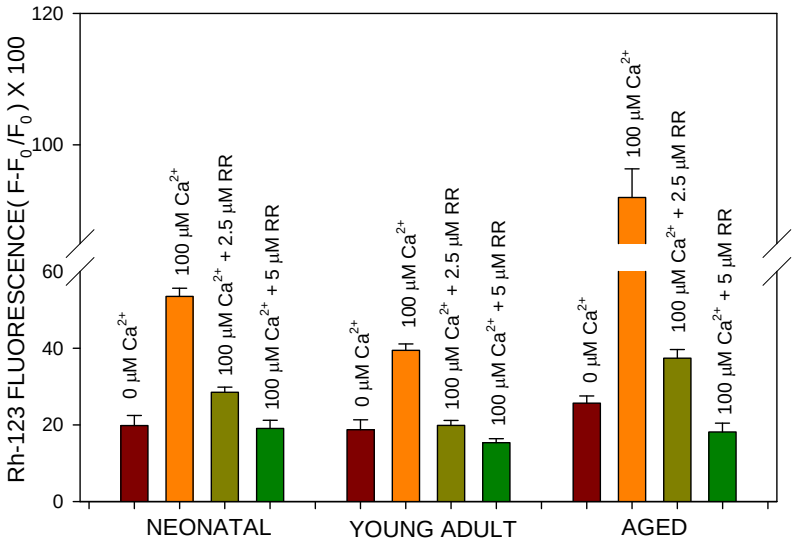

Fig. 2. Effect of ruthenium red (RR) on mitochondrial membrane depolarization in three different age. Alterations in membrane potential were measured by inhibiting Ca^{2+} uniporter with ruthenium red at two different concentrations (2.5 µM or 5 µM). The decrease in fluorescence indicates less depolarization observed as a result of inhibiting Ca^{2+} influx to mitochondria. Data points represent the mean ± SD of 6 experiments ($p < 0.001$).

Ca^{2+} DEPENDENT ROS GENERATION IN MITOCHONDRIA

The effect of extracellular calcium on reactive oxygen species generation was measured in mitochondria isolated from young adult rat brains. ROS formation was measured using a fluorescent probe-DCFH-DA. The mitochondria loaded with DCFH-DA showed an increase in fluorescence at 530 nm, indicating the oxidation of DCFH to DCF by reactive oxygen species. The mitochondria isolated from neonatal rat brains were treated with various calcium concentrations – 10 μm, 50 μm, 100 μm, 200 μm, 500 μm and 1 mM Ca^{2+}, and the formation of ROS were measured. The ROS formation for the various Ca^{2+} concentrations is given in Fig. 3. Increased ROS upon the increase in every bolus of Ca^{2+} concentration is indicated with increased DCF fluorescence.

The age-associated changes observed in ROS formation, quantified in mitochondria isolated from neonatal, young adult and aged rats is shown in Fig. 4. At 100 μM Ca^{2+} concentration, the three age groups showed characteristic increases in ROS generation. The mitochondria isolated from aged rat brains showed a 58.93 % increase in the formation of ROS as compared to neonatal and 53.13 % for young adult rats, respectively, in their basal rate of ROS production that was not exposed to Ca^{2+}. In the presence of 100 μM Ca^{2+}, mitochondria isolated from aged rats showed a 43.21 %-fold rise in ROS formation when compared to their control and 65.10 % when measured against ROS produced from neonatal rats.

EFFECT OF RUTHENIUM RED ON Ca^{2+} INDUCED MITOCHONDRIAL REACTIVE OXYGEN SPECIES GENERATION

Mitochondria accumulate Ca^{2+} through a uniporter, which ruthenium red effectively blocks. The mitochondria were incubated in 2.5 μM or 5 μM ruthenium red for 2 minutes, after which these ruthenium red inhibited mitochondria were challenged with a 100 μM dose of Ca^{2+}. Upon treating with 2.5 μM ruthenium red, there was 17.12 % restraint in ROS generation in the neonatal rats compared to their respective controls. However, in neonatal and young adult rat mitochondria, 21.78 % and 26.72 % inhibition were observed, respectively. In the presence of 5 μM ruthenium red, the mitochondria isolated from aged rats showed −1.83 % suppression in reactive oxygen species generation.

On the other hand, the mitochondria isolated from neonates and young adult rats exhibited −3.2 % and 1.54 % reduction in ROS generation for the same concentration, as shown in Fig. 4. These experiments are evident that Ca^{2+} plays a crucial role in ROS generation. However, the precise mechanism of its action in the generation of ROS needs further elucidation.

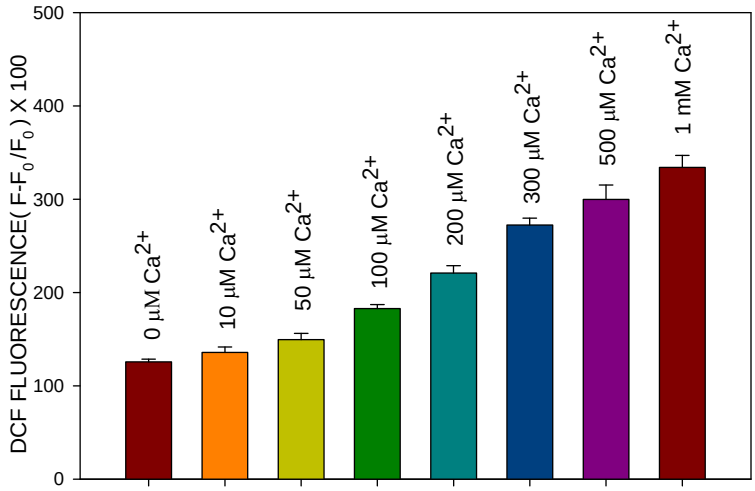

Fig. 3. Effect of Ca^{2+} on reactive oxygen species generation in mitochondria. Mitochondria were isolated from young adult rat brain. Data points represent the mean ± SD of 6 experiments ($p < 0.001$).

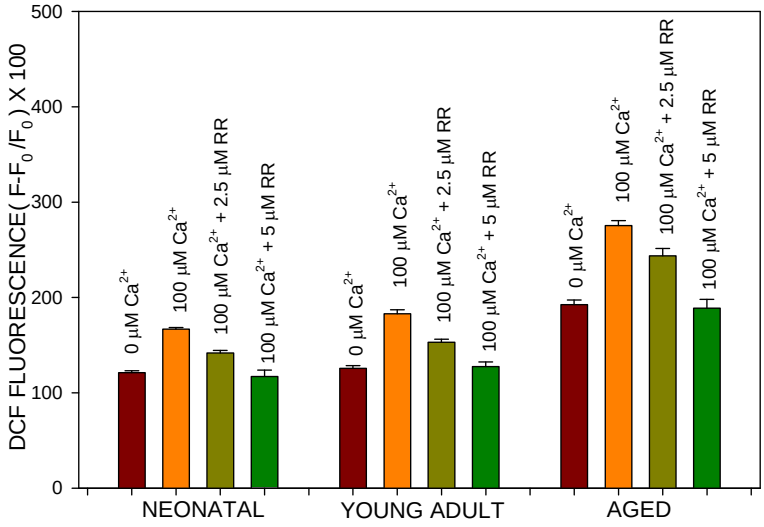

Fig. 4. Effect of ruthenium red on reactive oxygen species generation in three different age groups. DCFH-DA loaded mitochondria were incubated with 2.5 µM or 5 µM ruthenium red for 2 minutes and were Ca^{2+} challenged. ROS generated is measured in DCF fluorescence. The mitochondria of three different age groups treated with ruthenium red showed suppression in reactive oxygen species production. Data points represent the mean ± SD of 6 experiments ($p < 0.001$).

DISCUSSION

The present study was carried out in whole-brain mitochondria isolated from neonatal, young adult and aged rats to understand the role of Ca^{2+} in the alterations of the mitochondrial membrane potential, leading to the generation of ROS with aging. Isolated mitochondria is the best model to study specific mitochondrial functions under varied experimental conditions such as modulating Ca^{2+} uptake, its extrusion ways, and processes in specific inhibition of the respiratory chain complexes. It has been suggested earlier that the rise in mitochondrial Ca^{2+} could play a fundamental role in initiating the development of glutamate-mediated neuronal death derived from free radical generation [16]. Therefore, it was of interest to study the effect of Ca^{2+} concentrations on isolated mitochondria and understand the specific physiological variations that lead to oxidative stress.

Our experiments on increasing concentrations of Ca^{2+} have shown an increase in the formation of reactive oxygen species. Although the mechanism underlying the increased ROS production rate in response to Ca^{2+} is not clear [20], a previous view was that Ca^{2+} influx uncouples the proton gradient from ATP synthase, resulting in increased flux through the electron transport chain and therefore increased ROS production [7]. Our experiments on three age groups of rats have shown an increase in reactive oxygen species formation with age. However, the increase in ROS generation was more significant in the mitochondria isolated from aged rats. We observed an age-dependent increase in ROS production. Mitochondria isolated from aged rats untreated with Ca^{2+} have shown a small increase in their basal ROS generation compared to the two other age groups. The internal processes and reasons for the increase in ROS production during aging are still ambiguous. However, many studies have shown that the mitochondrial membrane stability and integrity declines as animals get old [12] and has been suggested that some inherent age-related differences could be responsible for the formation of ROS. External factors like cold elicited stress and several pesticide compounds are also known to decline mitochondrial activity with age [3].

Reactive oxygen species generation is linked to normal cellular processes, including cell metabolism, mitochondrial respiration, lipoxygenase, and cyclooxygenase activity, which may increase with age [15]. The increased ROS production by Ca^{2+} in mitochondria isolated from aged rats may be due to reduced antioxidant levels in the cell. Many enzymes, including superoxide dismutase, peroxidase, catalase, and glutathione peroxides, which protect ROS formation in the brain, are expressed at different levels during the early postnatal period and with an increase in age [27]. The antioxidant deficit could be one of the principal causes of the increased ROS formation observed in mitochondria isolated from aged rats.

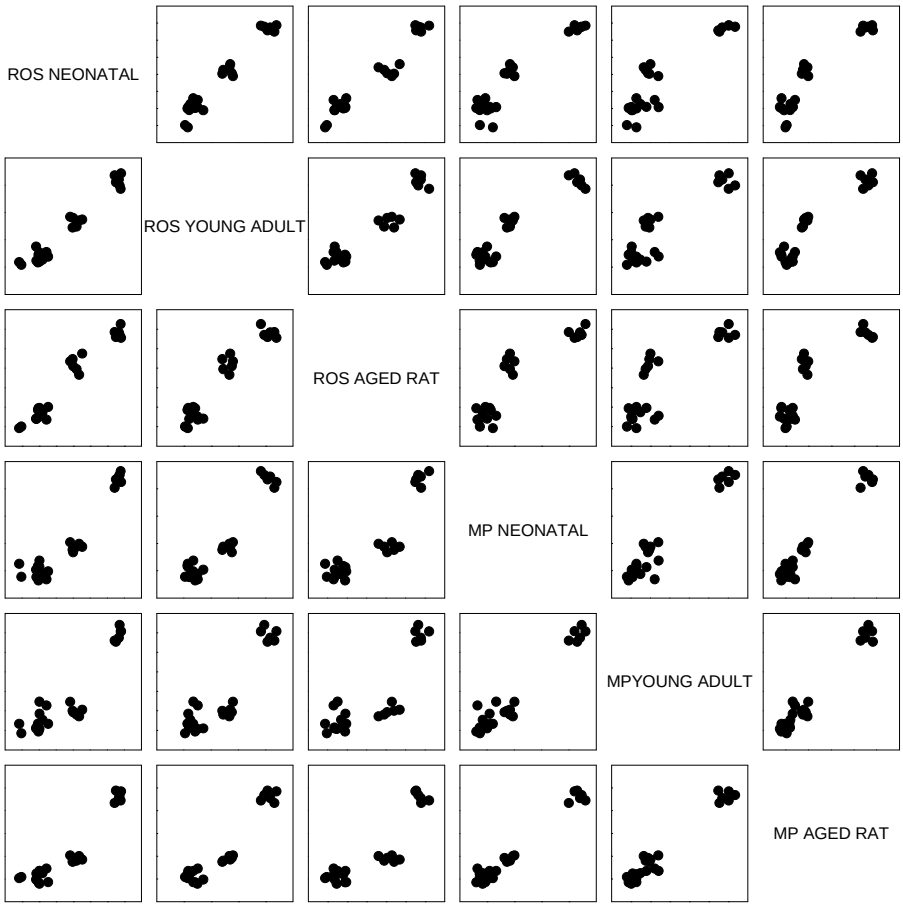

Fig. 5. Pearson Product Moment Correlation results indicate $p < 0.050$ in both membrane potential alteration and ROS generation rise together and show interdependency to the effect of Ca^{2+}. Note that the data points follow similar trend as membrane potential alteration and ROS generation falling in a straight line.

This study's findings indicate a rise in ROS formation with Ca^{2+} concentration, consistent with earlier studies performed on primary neuronal culture [16] and cortical slices of the brain [24]. The increase in ROS generation was observed in all three age groups, but the magnitude of ROS generated was different for each age. To further examine whether Ca^{2+} treatment alone has caused the ROS formation or Ca^{2+} uptake by mitochondria was responsible for the same, we incubated mitochondria with ruthenium red, a specific blocker of Ca^{2+} uniporter. Upon Ca^{2+} challenge to the ruthenium red treated mitochondria, there

was a drastic decrease in ROS production in all the three age groups. At a higher concentration of ruthenium red, mitochondria isolated from all the three age groups reduced by >95 % production in their reactive oxygen species formation. These experiments confirm the role of Ca^{2+} in reactive oxygen species generation. The results in this study suggest that mitochondria are the vital source of ROS production, is supported by many studies from other labs [2, 5, 6, 26]. Since the mitochondrial potential determines Ca^{2+} uptake, ATP synthesis, and other metabolic functions, we investigated the role of Ca^{2+} on membrane potential alterations.

The study results suggest a note-worthy increase in extramitochondrial Ca^{2+} depolarizing the mitochondrial membrane. The shift in mitochondrial membrane potential observed among all three age groups. In the absence of calcium in the medium, mitochondria isolated from aged rats showed a small depolarization. However, significant variations in mitochondrial depolarization observed among the three age groups upon Ca^{2+} treatment. Mitochondria isolated from aged rats showed more substantial depolarization of the membrane than neonatal and young adult rats. This result is consistent with previous work [9, 22].

Although the precise *raison d'être* behind the depolarized state of mitochondria as observed in neonatal age rats is unknown, it may be due to alterations in the respiratory capacity, and its debilitating effects on mitochondria during aging cannot be ignored. However, mitochondria isolated from young adult rats were the least depolarized. Our results are consistent with previous work done on cortical slices, supporting that in the neonatal age group, mitochondria were slightly depolarized than those in the young adult age groups. Studies on ruthenium red treated mitochondria showed reduced membrane depolarization in mitochondria of all three groups. At higher concentrations of ruthenium red, there was a complete decrease in membrane depolarization in mitochondria of all three age groups. These results strongly suggest that mitochondria accumulate Ca^{2+} avidly when local Ca^{2+} level in the surrounding are increased. Evidence from ruthenium red treatment [28] in isolated mitochondria also suggests that Ca^{2+} induced ROS production is interlinked to Ca^{2+} induced depolarization of the mitochondrial membrane. Pearson product moment correlates $p < 0.050$ addresses this interdependent relationship between ROS production and mitochondrial membrane potential alteration (Fig. 5). However, the mechanism of ROS formation and its increased production as a function of age in isolated mitochondria is still elusive to understand. Several studies on membrane potential changes in different age groups have indicated variations in the mitochondrial depolarization.

In the present study, we have demonstrated that reactive oxygen species formation and mitochondrial depolarization are highly dependent on mitochondrial Ca^{2+} uptake. Ca^{2+} buffering by mitochondria is very vital during a massive influx of Ca^{2+} through glutamate receptors. Ca^{2+} overload in the mitochondria leads to a

vicious cycle: an initial ROS-induced impairment of mitochondria leads to an increase in the production of oxidants, which in turn leads to further mitochondrial damage, subsequently affecting the whole cell's bioenergetics, leading to death.

CONCLUSIONS

Since a large increase in ROS production and increased depolarization of mitochondria isolated from aged rats was observed, it posed a question as to why the mitochondria isolated from aged rats are more vulnerable to Ca^{2+} stimulus. We believe a combination of elevated Ca^{2+} and mitochondrial functions may play a role in the above. Our data provide clear evidence that mitochondria isolated from aged rats (2–3 years old) are more vulnerable to the dissipation of membrane potential leading to an elevated ROS formation, suggesting that mitochondria are the target organelles.

It is tempting to view ROS generation as the only parameter of importance concerning mitochondria's role in oxidative stress. Indeed, as it follows from data and literature [2, 11], undermining several antioxidant indices is a precarious event overrating ROS formation by mitochondria. Otherwise, very little ROS is generated in the cytoplasm and elsewhere, including intra-mitochondrial sites. Cells with a battery of antioxidant defense mechanisms at their disposal still fail to combat the magnitude of ROS generated by mitochondria, are most likely seems to indicate 'defense failure' adequately describing the role of mitochondria in the onset of oxidative stress in aging. However, the mitochondrial role in oxidant-antioxidant balancing and aging needs further scientific enquiry.

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