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Time Dependent Ca^{2+} Induction Led to the Formation of Mitochondrial Permeability Transition Pore as a Function of Age

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Abstract:- Ca^{2+} sequestration and its homeostasis is disrupted when mitochondrial membrane permeabilises to form a large opening - the mitochondrial permeability transition pore (MPTP). The MPTP formation is common during aging and age related pathologies, activating cell death pathways to avoid unhealthy consequences and malignancies in the brain tissue. Several studies have identified the participation of Cyclophilin-D (Cyp-D) and adenine nucleotide translocase(ANT) in forming MPTP. However, Ca^{2+} is known to participate significantly in MPTP induction, although its concentration and time dependent permeabilization mechanisms are still elusive.

In this work, we have focused on the contribution of Ca^{2+} participation, its concentration and time taken to permeabilze the mitochondrial inner membrane as a measure of light scattering at 540 ηM. We have observed that MPTP formation is increased in mitochondria isolated from aged rats in comparison to young adult and neonatal rats. The cyclosporin A application blocks the cyclophilin interaction thus avoiding MPTP formation confirming the crucial role of Ca^{2+} inducted MPTP opening. A 100 µM Ca^{2+} incubation for 15 minutes allowed the 50% probability of formation of MPTP in mitochondria isolated from all age groups. Thus, alleviating its role in aging and neurodegeneration.

Keywords:- *Mitochondria;Ca^{2+};Aging;Mitochondrial Permeability Transition Pore (MPTP).*

I. INTRODUCTION

Mitochondria is a very active and dynamic organelle constantly participating in sequestration of several ions, and most importantly Ca^{2+}. Ca^{2+} handling in the brain is of particular importance to us, as it plays innumerable roles such as membrane depolarization, synaptic plasticity [1], memory formation, programmed cell death [2], oxidative stress [3], aging and age related diseases [4], [5]. Mitochondria are known to uptake and or extrude when surrounding cytoplasmic Ca^{2+} concentration fluctuates [6]. The surplus Ca^{2+} increase in the medium would allow mitochondria to avidly take up Ca^{2+} alleviating its unregulated effects on the cell. However, when Ca^{2+} crosses more than the threshold, mitochondria are known to swell up, due to osmotic shock [7]. An array of ions in the media contributes to varied osmolarities to induct swelling in mitochondria.

Ca^{2+} is known to intrude in several functions of mitochondrial physiology. However, the mitochondrial membrane exposed to various ultrastructural changes appears to be the focal point of swelling. Imaging studies reveal variation in histoarchitecture of mitochondrial cristae intervening in oxidative phosphorylation and ATP production[8]. Mitochondrial swelling allows the mitochondrial membrane to dilate and allow the passage of molecules of size less than 1500 Da in molecular weight [7]. This is due to the configuration of pore-forming protein in the inner membrane called mitochondrial permeability transition pore (MPTP or mPTP; also known widely as PTP, mTP, or MTP). MPTP opening is common in neuro-pathological conditions such as stroke, brain injury, Parkinson's disease, and Alzheimer's disease [9]. The induction of MPT is known to result in mitochondrial swelling and neuronal death through programmed cell death mechanism or necrosis depending on the neurological setting. MPTP is also common in Ca^{2+} dysregulation leading to excitotoxicity, wherein over activation of glutamate receptors would load up cells with surplus Ca^{2+} creating havoc and cell death [10]. This is possible due to Ca^{2+} binding and activating matrix side of the MPTP. MPTP can result due to the excessive alteration in mitochondrial transmembrane potential, leading to varied voltage fluctuations across the mitochondrial inner membrane [11]. However, in brain tissue, MPTP induction is complicated to understand and delineate the contribution of membrane potential. Altered membrane potential would skew ATP production and begin consuming more ATPs. This creating an energy demand situation that reduces the activity of Ca^{2+} exchangers leading to energy-deprived conditions [12]. Moreover, oxidative stress due to multiple factors can also trigger off MPTP opening. Ca^{2+} seems to be playing a double role here in eliciting oxidative load on the cell and simultaneously deregulating mitochondrial membrane potential. More so, a surplus load of Ca^{2+} can energize mitochondria, and push the same to swell, resulting in MPTP induction.

Aging, though multi-factorial in origin, and show signs of deterioration in physical and behavioral wellbeing of an organism, exhibit mitochondrial dysfunction at the cellular level. Mitochondria are intimately involved in aging as it sequesters Ca^{2+}, increases free radical generation, MPTP opening, apoptosis, and ATP production as well. In a study done by Mather and Rottenberg, aging is known to enhance mPTP opening [13]. Contrary to this idea, MPTP induction can be a prospective target for intervention of new drugs against neurodegenerative

disease, central nervous system dysfunction, injury, trauma, and stroke [12].

Molecules like glutathione can exit mitochondria when MPTP is inducted resulting in reduced antioxidant defense. Besides, enzyme complexes of electron transport chain may produce copious amounts of free radicals triggering MPTP and resulting in the loss of components like cytochrome c [14], [15]. More so, MPTP can also participate in Ca^{2+}-dependent proteases like calpain activation.

MPTP measurement is very simple as it involves a large decrease in light scattering absorbance at 540nm, which can be partially upturned by the chelation of Ca^{2+}. MPTP can also be blocked transiently using immune suppressant drugs like cyclosporin A (CsA) and N-methyl-Val-4-cyclosporin A (MeValCsA), and non-immunosuppressants like EGTA, EDTA, and several derivates of CsA as well. Despite these important findings, several questions concerning the MPTP have remained elusive during aging.

Our study aims to address the role of Ca2+ in MPTP formation in relation to aging. Ca2+ dysregulation is often seen in aged and neurodegenerative pathologies. Hence, investigation on the time-dependent effect of Ca2+ on MPTP formation should unveil the problems associated with studying MPTP formation in isolated mitochondria. Thus, aiding a better understanding of the subject, while deliberations and interpretations were made on isolated mitochondria in aged conditions.

II. MATERIALS AND METHODS

A. Materials

Ca^{2+}, HEPES, Tris- HCl, Sucrose, Succinate, P_i, EGTA were of analytical grade and bought from BDH chemicals. Alamethicin, Cyclosporin A, and Rotenone were procured from Sigma Chemicals, USA.

B. 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 standard differential centrifugation method as described by [16] 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 tetra potassium EDTA,75 mM sucrose, and20 mM 0.1% fatty acid-free bovine serum albumin (BSA). The tissue was chopped into small pieces, minced and ground to form a homogenate, which was mixed with the buffer in a 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 spun at 20,000 rpm for 20". The mitochondria collected as pellets were treated with storage buffer similar to isolation buffer, except that it contained 0.1mM 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 -40^0C. All the isolation procedures and methods were carried out at $0\text{-}4^0$C to retain the maximal activity of the isolate. The total protein was concentrated to 1mg/mL in storage buffer upon Lowry's method of estimation.

- *Mitochondrial marker assay*

Succinate dehydrogenase (SDH) of the electron transport chain is a marker enzyme of mitochondria and entails to check the health of the sample isolated as well. It is an integral membrane protein and its disruption distorts the mitochondrial membrane architecture. The SDH assay uses succinate as a substrate and 2,6-dichlorophenolindophenol (DCPIP) as an electron acceptor [17]. All three age groups of SDH activities were measured for statistically significant results to indicate their viability (data not shown).

Mitochondrial Permeability Transition (MPT) measurements of isolated rat brain mitochondria

Treatment of Ca^{2+} induces osmotic swelling of freshly isolated rat brain mitochondria suspensions forming mitochondrial permeability transition pore. This swelling was routinely measured by reading absorbance at 540 ηM in a spectrophotometer for 30 min at room temperature. To 1 ml final assay volume, containing mitochondria at 100 µg/ml in "standard swelling buffer" (20 mM HEPES, 10mM Tris- HCl, 200 mM Sucrose, 5 mM Succinate, 1 mM P_i, 10 µM EGTA and 15 µM Rotenone; pH 7.4) treated with variable concentration of Ca^{2+} ranging from (zero) 0 µM to 300 µM and their decreasing absorbance were noted down. Alamethicin as an MPT-inducing agent was used at 0.1 µg/100 µg mitochondrial protein when required. Isolated rat brain mitochondria were treated with a high dose of Ca^{2+} (300 µM) to ensure complete swelling. We investigated for a relationship between optical density readings and mitochondrial membrane permeabilization with and without Ca^{2+} of variable concentrations. Time taken to completely swell mitochondria was also noted down. Cyclosporin A is commonly known inhibitor of MPT opening. 2.5 µM and 5 µM CsA was added separately in each set of experiment, 4 minutes before Ca^{2+} application to inhibit MPT opening. Ruthenium red is fraught with interference at 540 ηM and hence Cyclosporin A is being employed as a better substitute for blocking MPT opening.

- *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-value of <0.001 as significant.

III. RESULTS

Ca^{2+} treatment induces mitochondrial swelling. Ca^{2+} concentration of 0µM to 300µM treatment resulted in various levels of membrane permeabilization. The degree of membrane permeabilization is directly related to the value of absorbance decreased at 540nM. Steady-state of absorbance indicated the membrane stability whereas decreasing values of light scattering implied mitochondrial swelling. Our study utilized three age groups of *Sprague dawley* rats viz 2-3 weeks old (neonatal), 2-3 months old (young adult), and 2-3-year-old (aged) rats. A comparison among the 3 age groups has revealed the rate of membrane permeabilization and its functional significance.

- *Ca^{2+} induced mitochondrial swelling in neonatal rats*

Various concentrations ranging from 0 to 300µM Ca^{2+} were treated to mitochondria isolated from neonatal rats (Fig.1). Ca^{2+} untreated mitochondria followed steady-state kinetics for 30 minutes showed very less membrane permeabilization of <12% (Table 1). However, 100µM Ca^{2+} challenge for 15 minutes showed ~50% permeabilization signifying membrane viability, stability, and integrity to be 50%. 300µM Ca^{2+} treatment though showed ~50% permeabilization at 15th minute, winded up in rapidly dropping its absorbance values confirming MPTP opening. Our experiment with 100µM Ca^{2+} along with MPTP blocker Cyclosporin A resulted in ~12% mitochondrial swelling, which is in line with Ca^{2+} untreated mitochondria or control. Detailed change in percentage of permeabilization upon Ca^{2+} treatment can be accessed from the table for all the age groups.

- *Ca^{2+} induced mitochondrial swelling in young adult rats*

Mitochondria isolated from young adult rats followed a similar trend as of neonates when Ca^{2+} of various concentrations was treated (Fig.2). 0µM Ca^{2+} challenge resulted in ~12% mitochondrial swelling, whereas 300µM Ca^{2+} treatment for 30 minutes resulted in >80% membrane permeabilization (Table 2). 100µM Ca^{2+} addition resulted in a ~50% decrease in absorbance values and upon Cyclosporin A treatment reduced the swelling to <5% in 15-minute duration as in the case of control.

- *Ca^{2+} induced mitochondrial swelling in aged rats*

Mitochondria isolated from aged rats indicate a little dilated and fast permeabilizing trend upon Ca^{2+} treatment (Fig.3). Ca^{2+} untreated mitochondria exhibit about 15+ % mitochondrial swelling whereas 300µM Ca^{2+} treatment brought out 80% membrane permeabilization representing the possibility of fast MPTP opening (Table 3). However, 100µM Ca^{2+} treatment brought about <50% membrane swelling and Cyclosporin A addition further reduced the swelling rate to ~16%.

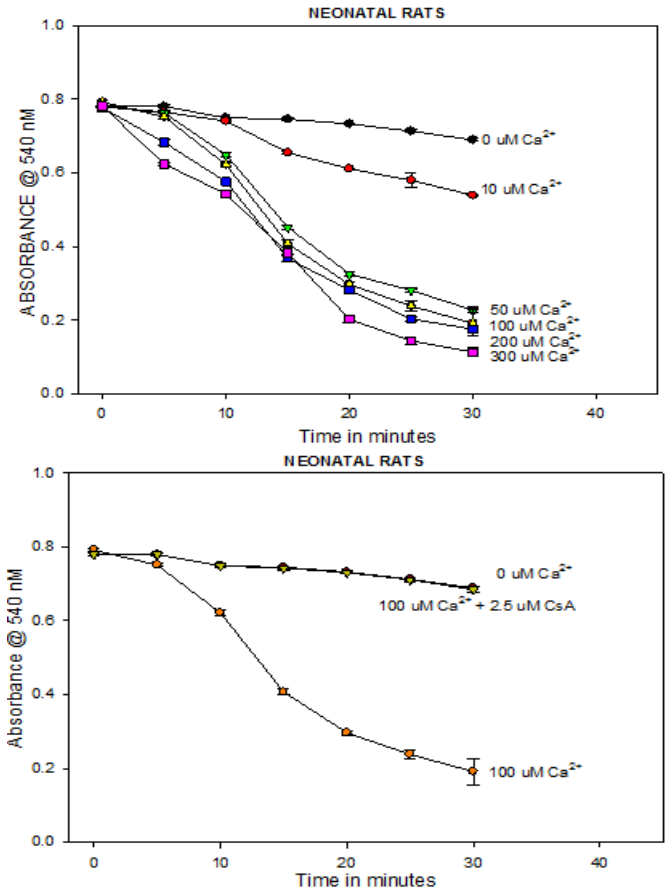

Fig 1:- Absorbance measured at 540nm against the different concentrations of Ca2+(from 0µM to 300 µM) in 5 minutes time intervals of 30 minutes duration. The lower absorbance value indicates the higher membrane permeabilization and swelling. Addition of 2.5 µM CsA completely suppresses the MPTP opening.

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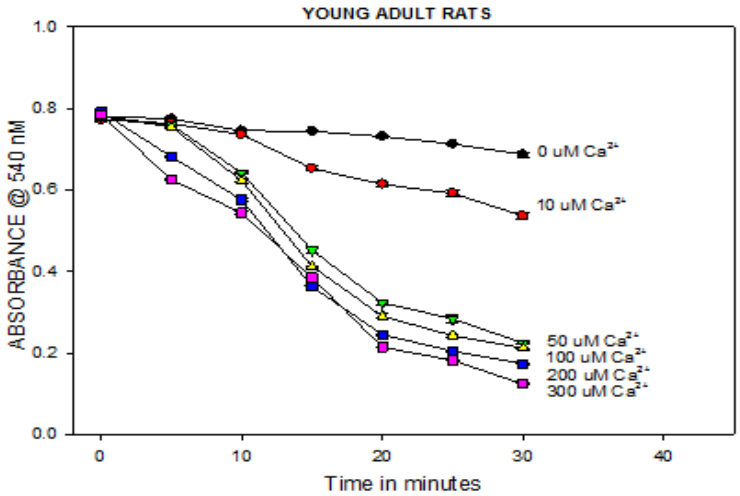
NEONATAL RATS	0 min	5 min	10 min	15 min	20 min	25 min	30 min
0 µM Ca^{2+}	0.781± 0.004	0.780± 0.004	0.749± 0.002	0.745± 0.003	0.733± 0.001	0.713± 0.004	0.689± 0.004
% of decreasing absorbance		-0.192	-4.140	-4.588	-6.210	-8.728	-11.610
10 µM Ca^{2+}	0.781± 0.006	0.764± 0.002	0.740± 0.002	0.655± 0.004	0.611± 0.002	0.579± 0.020	0.539± 0.002
% of decreasing absorbance		-2.110	-5.168	-16.143	-21.781	-25.774	-29.519
50 µM Ca^{2+}	0.780± 0.002	0.762± 0.001	0.649± 0.007	0.452± 0.006	0.325± 0.007	0.280± 0.005	0.227± 0.007
% of decreasing absorbance		-2.251	-16.789	-42.027	-58.358	-64.044	-70.236
100 µM Ca^{2+}	0.792± 0.004	0.751± 0.004	0.622± 0.006	0.407± 0.009	0.297± 0.006	0.238± 0.012	0.191± 0.036
% of decreasing absorbance		-5.154	-21.507	**-48.569**	-62.563	-69.949	-74.529
200 µM Ca^{2+}	0.776± 0.011	0.682± 0.007	0.575± 0.008	0.368± 0.009	0.281± 0.007	0.203± 0.007	0.175± 0.009
% of decreasing absorbance		-12.113	-25.881	-52.620	-63.789	-73.883	-74.316
300 µM Ca^{2+}	0.781± 0.007	0.623± 0.005	0.541± 0.009	0.383± 0.007	0.202± 0.009	0.141± 0.009	0.115± 0.012
% of decreasing absorbance		-20.294	-30.794	-50.960	-74.129	-82.010	-81.606
100 µM Ca^{2+} + 2.5µM CsA	0.780± 0.003	0.780± 0.004	0.749± 0.003	0.743± 0.005	0.731± 0.003	0.711± 0.007	0.685± 0.009
% of decreasing absorbance		-0.064	-3.996	-4.744	-6.303	-8.803	-12.123

Table 1: The table indicates the isolated mitochondria being treated with various concentrations of Ca2+viz 0 to 300µM over duration of 30 minutes and an interval of 5 minutes between each recording. Values are Mean ± SD of six replicates represented as the decreasing absorbance indicating MPTP opening in mitochondria isolated from neonatal rat brain.



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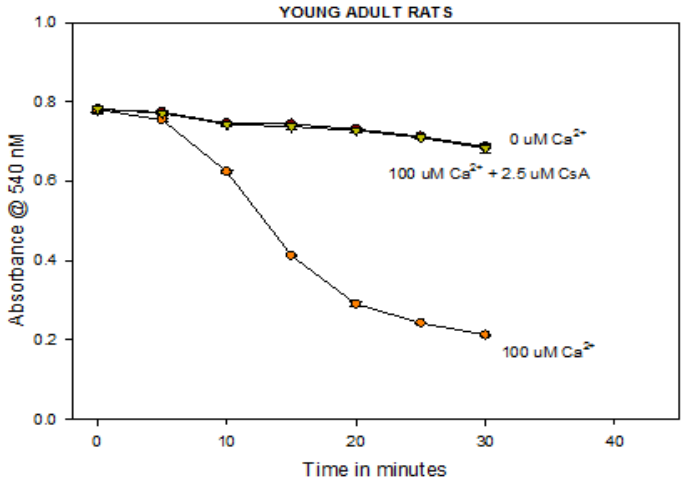

Fig 2:- Absorbance measured at 540ηM against the different concentrations of Ca2+ (from 0µM to 300µM) in 5 minutes time intervals of 30 minutes duration. The lower absorbance value indicates the higher membrane permeabilization and swelling. Addition of 2.5 µM CsA completely suppresses the MPTP opening

YOUNG ADULT RATS	0 min	5 min	10 min	15 min	20 min	25 min	30 min
0 µM Ca^{2+}	0.782± 0.006	0.774± 0.004	0.746± 0.003	0.744± 0.002	0.731± 0.003	0.713± 0.003	0.687± 0.006
% of decreasing absorbance		-0.959	-4.606	-4.797	-6.439	-8.806	-11.259
10 µM Ca^{2+}	0.773± 0.007	0.763± 0.005	0.736± 0.007	0.653± 0.006	0.614± 0.005	0.592± 0.006	0.537± 0.008
% of decreasing absorbance		-1.223	-4.725	-15.469	-20.539	-23.387	-29.690
50 µM Ca^{2+}	0.779± 0.005	0.760± 0.006	0.642± 0.003	0.452± 0.006	0.324± 0.004	0.284± 0.010	0.225± 0.003
% of decreasing absorbance		-2.457	-17.652	-41.980	-58.490	-63.601	-70.468
100 µM Ca^{2+}	0.780± 0.009	0.755± 0.003	0.623± 0.003	0.412± 0.002	0.290± 0.006	0.243± 0.003	0.213± 0.002
% of decreasing absorbance		-3.289	-20.141	**-47.159**	-62.794	-68.881	-71.842
200 µM Ca^{2+}	0.792± 0.001	0.682± 0.001	0.576± 0.002	0.363± 0.003	0.245± 0.002	0.205± 0.002	0.174± 0.002
% of decreasing absorbance		-13.874	-27.242	-54.147	-69.011	-74.168	-74.529
300 µM Ca^{2+}	0.785± 0.002	0.625± 0.002	0.543± 0.003	0.385± 0.002	0.214± 0.002	0.183± 0.001	0.124± 0.002
% of decreasing absorbance		-20.395	-30.848	-50.988	-72.721	-76.737	-80.224
100 µM Ca^{2+} + 2.5µM CsA	0.782± 0.008	0.772± 0.007	0.743± 0.006	0.738± 0.007	0.728± 0.004	0.710± 0.005	0.683± 0.010
% of decreasing absorbance		-1.343	-4.966	-5.691	-6.905	-9.271	-11.471

Table 2:- The table indicates the isolated mitochondria being treated with various concentrations of Ca2+viz 0 to 300µM over duration of 30 minutes and an interval of 5 minutes between each recording. Values are Mean ± SD of six replicates represented as the decreasing absorbance indicating MPTP opening in mitochondria isolated from brain tissue of young adult rat.

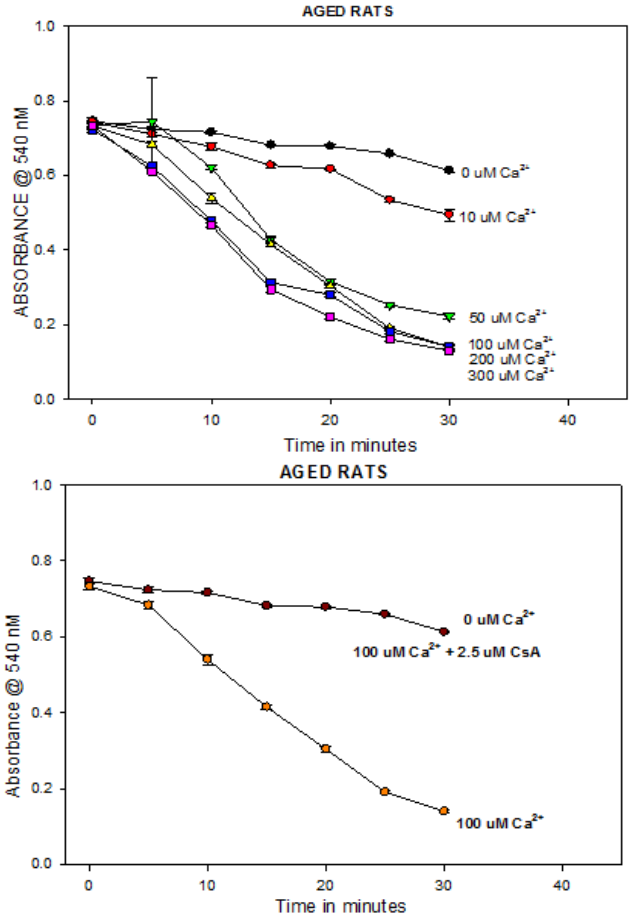

Fig 3:- Absorbance measured at 540ηM against the different concentrations of Ca^{2+}(from 0µM to 300µM) in 5 minutes time intervals of 30 minutes duration. The lower absorbance value indicates the higher membrane permeabilization and swelling. Addition of 2.5 µM CsA completely suppresses the MPTP opening

AGED RATS	0 min	5 min	10 min	15 min	20 min	25 min	30 min
0 µM Ca^{2+}	0.746± 0.009	0.724± 0.007	0.717± 0.004	0.682± 0.003	0.678± 0.004	0.659± 0.002	0.613± 0.003
% of decreasing absorbance		-3.039	-3.997	-8.642	-9.111	-11.657	-15.291
10 µM Ca^{2+}	0.743± 0.006	0.711± 0.006	0.678± 0.012	0.627± 0.006	0.618± 0.002	0.535± 0.007	0.494± 0.016
% of decreasing absorbance		-4.329	-8.793	-15.568	-16.891	-28.062	-30.457
50 µM Ca^{2+}	0.735± 0.008	0.745± 0.119	0.621± 0.007	0.428± 0.011	0.316± 0.008	0.253± 0.007	0.222± 0.008
% of decreasing absorbance		1.361	-15.447	-41.742	-57.033	-65.585	-70.166
100 µM Ca^{2+}	0.733± 0.009	0.683± 0.009	0.540± 0.013	0.415± 0.007	0.304± 0.007	0.190± 0.005	0.140± 0.005
% of decreasing absorbance		-6.756	-26.342	**-43.403**	-58.508	-74.022	-79.507
200 µM Ca^{2+}	0.724± 0.007	0.625± 0.005	0.479± 0.007	0.313± 0.011	0.280± 0.006	0.182± 0.006	0.141± 0.007
% of decreasing absorbance		-13.614	-33.840	-56.692	-61.345	-74.868	-77.493
300 µM Ca^{2+}	0.732± 0.009	0.611± 0.007	0.469± 0.006	0.298± 0.009	0.223± 0.006	0.159± 0.007	0.131± 0.005
% of decreasing absorbance		-16.473	-35.954	-59.330	-69.583	-78.264	-78.642
100 µM Ca^{2+} + 2.5µM CsA	0.741± 0.006	0.721± 0.006	0.713± 0.007	0.677± 0.009	0.659± 0.008	0.647± 0.009	0.604± 0.009
% of decreasing absorbance		-2.746	-3.868	-8.658	-11.154	-12.683	-16.252

Table 3:- The table indicates the isolated mitochondria being treated with various concentrations of Ca^{2+}viz 0 to 300µM over duration of 30 minutes and interval of 5 minutes between each recording. Values are Mean ± SD of six replicates represented as the decreasing absorbance indicating MPTP opening in mitochondria isolated from brain tissues of aged rats.

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IV. DISCUSSION

The current study was pursued to clarify the role of surplus Ca^{2+} in distorting mitochondrial membrane architecture in relation to aging. Permeabilization of the mitochondrial membrane is a significant step in understanding the pathophysiological state of the cell; to assess whether the cell would be pushed to apoptosis and/or necrosis. Ca^{2+} takes in various roles in the cell, as a secondary messenger to death elicitor and thus making Ca^{2+} sequestration to be a tightly regulated physiological phenomenon in the cell. The molecular weight of solutes ≤1.5 kDa can easily pass through the mitochondrial membrane when surplus Ca^{2+} increases the permeability of the inner membrane forming pores. These pores are nonselective openings on the membrane and are called mitochondrial permeability transition pore (MPTP). The molecular assembly, structure, and MPTP's true identity are still elusive though. The abnormal accumulation of intramitochondrial Ca^{2+} and ROS release are mutually inclusive in the loss of membrane potential and altered oxidative phosphorylation resulting in declining ATP synthesis. ATP level declines in the cell as ATP synthase works in reverse mode hydrolyzing ATP and uncoupling mitochondria. The depleting of ATPs in the cell increases the chances of failure in cellular metabolism. Necrosis is also a strong outcome of ATP depletion. Thus, MPTP is known to play an innumerable role in aging and age related diseases of nondividing cell types like neurons and cardio myocytes.

Like aging, MPTP opening is multifactorial and involves hampered Ca^{2+} sequestration, elevated oxidative burden, increased cyclophilin D (Cyp-D) protein, denatured cardiolipins, and oxidative stress. Some evidence points out the vulnerability of mitochondria to Ca^{2+}-induced MPTP gaping to be greater in older animals compared to younger ones' brain [13] and cardiac tissues [18], [19]. Besides, the mitochondrial membrane is also involved in signals eliciting programmed cell death in response to stress on mitochondria. The mitochondrial inner membrane is selectively permeable to certain metabolites and ions. However, surplus accumulation of Ca^{2+} along with elevated levels of ROS release, combined with certain ambiguous factors and reduced adenine nucleotide pool in matrix induce the formation of nonspecific MPTP in the inner mitochondrial membrane [14], [20]–[22].

The current study focusing on Ca^{2+} treatment of various concentrations to the mitochondria isolated from three different age groups found increasing permeabilization of the inner mitochondrial membrane in samples of aged rats in comparison to young adults and neonatal rats (Fig.4 and Table 4 evaluating the percentage of significance). We found the decreased absorbance in relation to time as an indicator of the level of mitochondrial membrane swelling or MPTP opening. Our results clearly identify the role of increasing intramitochondrial Ca^{2+} as the causative factor of mitochondrial swelling. Our results are in agreement with Mather and Rottenberg who demonstrated mitochondria isolated from brain and liver of aged rats confirming a lower threshold for Ca^{2+}- elicited MPTP opening which is sensitive to cyclosporine treatment [13]. Furthermore, other tissue cells like cardio myocytes of older animals exhibited Ca^{2+} sensitive MPTP opening too [19], [23]. However, Cyclosporin A treatment drastically reduced the possibility of Ca^{2+} induced MPTP formation. This clearly signifying the role of elevated levels of Ca^{2+} influx into the mitochondria to be the cause of MPTP formation.

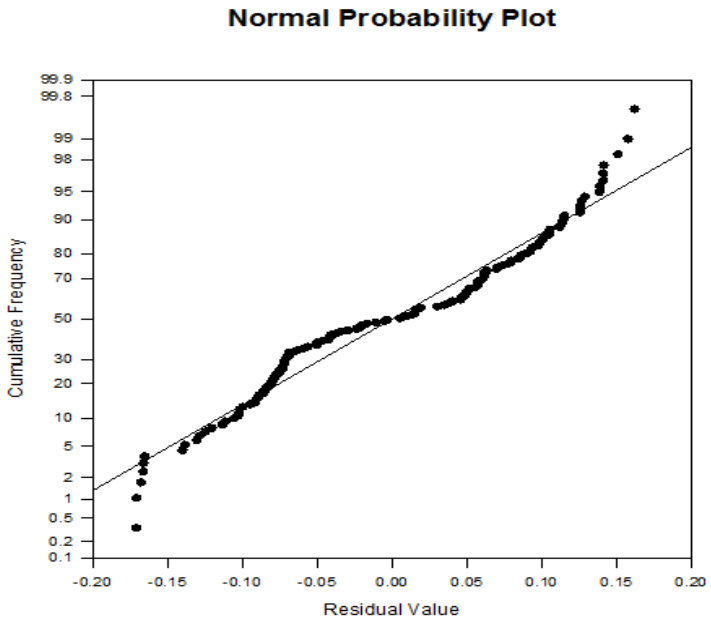

Fig 4:- Normal distribution showing more variance than the expected as seen in the curve starting below the normal line and ending above with long tail with $p<0.001$.

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Source of Variation	DF	SS	MS	F	P
AGE	2	0.0541	0.0271	106.651	<0.001
CAL CONC	6	2.577	0.430	1692.863	<0.001
TIME	6	3.199	0.533	2101.135	<0.001
Residual	72	0.0183	0.000254		
Total	146	6.968	0.0477		

Table 4:- The difference in the mean values among the different levels of age are greater than would be expected by chance after allowing for the effects of differences in age, calcium concentration and duration of experiment - time. There is a statistically significant difference $p<0.001$.

A 100uM Ca^{2+} treatment to isolated mitochondria showed a 50% formation of MPTP in 15 minutes of incubation. This time dependency is debatable and can be varied in the *in vivo* situation, where Ca^{2+} entry into the mitochondria gets diluted with ER and cytoplasm intervention. However, mitochondria of the brain seem to be acting as a large volume of Ca^{2+} sucker than other organelles in the same tissue. Additionally, cellular stress observed in the endoplasmic reticulum can induce Ca^{2+}-dependent MPTP opening in mitochondria initiating apoptosis [24]. However, the aged animals displaying increased vulnerability to MPTP opening is still a subject of debate till date. MPTP opening resulting from disturbed Ca^{2+} homeostasis is a one-dimensional perspective [25] while the increased oxidative load on the mitochondria can be an alternative and/or cumulative phenomenon leading to the formation of MPTP. Increased ROS release in aged animals is well recorded and verified by several researchers around the globe [4], [26], [27], though the link between MPTP opening and Ca^{2+} interplay is still obscure. However, the increased oxidative burden is expected to cause alteration in the form and function of biomolecules like proteins and lipids that are part of the major components of the mitochondrial inner membrane. In addition to alteration in Ca^{2+} and redox state which regulates MPTP opening, changes in protein expression and structural association with MPTP occur with aging. An elevation in the levels of the ratio between Cyclophilin-D (Cyp-D) and adenine nucleotide translocase(ANT) was identified to increase the vulnerability of the inner mitochondrial membrane to form MPTPs [28]. ANT is a commonly oxidized protein most often observed in aged animals [29]. ANT along with Cyp-D is considered to be components that form MPTP [14], [30]. Under conditions of surplus Ca^{2+} load and ROS release, the ANT reverses the flux between ADP and ATP and tends to denature and deform from its native state as a gated pore, into a non-selective channel. This result in small metabolites and ions that are ≤1500Da can traverse across the inner mitochondrial membrane without any hindrance. Increased free radical formation with age may be one of the reasons for ANT deformation and non-selectivity. Structurally ANT housing 3 redox-sensitive cysteine residues in the polypeptide chain extending as loops onto the mitochondrial matrix are vulnerable to oxidation in situations of low intra-mitochondrial GSH/GSSG ratio. Indeed, GSH/GSSG ratio is found to be considerably reduced in aged rats [31].

ANT's are also prone to be affected by oxidation of lipid components of membrane-like polyunsaturated fatty acids and thus, releasing fragment of membranes such as HNE that interact rapidly with thiol groups of ANT making it dysfunctional or reversing the ADP-ATP exchange across the membrane. Indeed, Kristal et.al., have reported 4-hydroxy-2-nonenal (HNE) to be a potent oxidant to initiate an MPTP opening [32]. Lipid composition, especially cardiolipin of the inner mitochondrial membrane is also known to vary with increased susceptibility to Ca^{2+} induced MPTP induction in aged animals [19]. In physiological conditions, cardiolipins are bound to ANTs for their optimal activity [33]. However, cardiolipin depletion deactivates ANT activity and deforms it to be a nonselective channel forming protein complex of MPTP. Thus, cardiolipin interacting with ANT and Ca^{2+} dyshomeostasis together act synergistically in MPTP induction. Our study, implicating Ca^{2+} in MPTP formation is supported by Ca^{2+} binding to the –vely charged cardiolipin head group participating in the disruption of the interaction between ANT and itself, thus allowing a structural modification in cardiolipin interacting with ANT to form a non-selective MPTP [34]. Cardiolipin oxidation can also destabilize ANT structure by breaking the active ANT dimerization [35].

Another important component of pore formation is Cyclophilin-D, which is a mitochondrial matrix protein known to interact with ANT to elicit the MPTP formation [36]. The reduced GSH/GSSG ratio in older animals, reducing antioxidant capacity significantly while allowing increased ROS production due to Ca^{2+} interplay has largely been known to form MPTP [37]. In brain tissue, glutamate toxicity extending to delayed Ca^{2+} deregulation resulting in neuronal death is known to be due to Cyp-D induced MPTP formation [38]. Decreased expression of Cyp-D in aged animals prevents the cells from committing suicide [39].

The current study aimed at realizing the role of Ca^{2+} in MPTP formation in the mitochondria isolated from young adult and neonatal brain tissue displayed 50% inhibition for 100 µM Ca^{2+} treatment for duration of 15 minutes. We observed a graded decrease in light scattering to each dose of increasing Ca^{2+} concentration treatment to the isolated mitochondria, confirming the opening of MPTP. This time-dependent permeabilization may be due to ionic concentration differences settling down to equilibrium across a membrane. The current study employed MPTP measurement should indicate the absorbance observed in elevated Ca^{2+} treatment and duration of time consumed to assess the probability and percentage of MPTP formation.

We found 30 minutes of incubation to have permeabilized the mitochondrial membrane significantly. The 300 µM Ca^{2+} treatment exhibiting more or less 80% MPTP opening in all three age groups is an indication of complete permeabilization at 30th minute and it can be significantly harmful to the cell. In an intact mitochondrion, Ca^{2+} effect would not last this long, as cell would orchestrate a coordinated homeostatic signals to elicit protective and counteractive mechanisms to avoid the danger of cell committing suicide or be part of Ca^{2+} excitotoxicity. However, isolated mitochondria lack this advantage and is prone to the excessive Ca^{2+} load allowing a researcher to investigate the role of MPTP formation in aging and neurodegenerative diseases as well. Thus, Ca^{2+} insult on the mitochondrial membrane has certain deleterious effects on the inner mitochondrial membrane starting from MPTP opening to disturbed ionic homeostasis and apoptosis initiation.

V. CONCLUSION

In summary, deranged Ca^{2+} homeostasis, elevated levels of Cyp-D, escalated oxidative load, oxidized ANT, may play a role in increased MPTP sensitivity with aging and age-associated diseases. The study is limited in not elaborating structural changes in MPTP formation, but do investigate the mitochondrial permeabilization with light scattering technique for measurement. The study confirms the 15-minute incubation of 100 µM Ca^{2+} induced MPTP formation to be nearly 50% in all three age groups. However, a better comprehension of the factors controlling the MPTP form and function may address the molecular mechanisms underlying aging and neurodegenerative pathologies. MPTP induction reversal can be a plausible pharmacological target and aid further in extending senescence.

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