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ORIGINAL ARTICLE

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Ca^{2+} induced Lipid Peroxidation Accentuates Aging in Rat Brain Mitochondria

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Aging in rats is multifactorial and include decline in biological, physiological, psychological and social changes. The antioxidant imbalance is one of the commonest factors during aging and age-related diseases. Several studies have identified the participation of various antioxidant's differential activity levels in the brain. However, Ca^{2+} is known to alter the homeostatic balance of these antioxidants. Lipid peroxidation is the chief marker of membrane architecture disruption in the aging and neurodegenerative diseases. In the current study, we have focused on the contribution of Ca^{2+} participation, its concentration and effect on the lipid peroxidation at various stages of rat's life. We have observed that LPO induction is increased in mitochondria isolated from aged rats in comparison to young adult and neonatal rats. Treatment of 100 to 300uM Ca^{2+} has shown a steady increase in lipid peroxidation of up to 20% in mitochondria isolated from aged rats while neonates and young rats have displayed differential levels of peroxidation from 17% to 15%. Thus, alleviating role of Ca^{2+} in lipid peroxidation in aging and neurodegeneration.

Key words: Lipid peroxidation, calcium, aging, rat brain, mitochondria

Aging has been ascribed to changes at molecular intersections and impairments due to free radical generation (Harman, 1956), alterations in immunity (Hong *et al.*, 2019; Martínez de Toda *et al.*, 2019), telomere shortening (López-Otín *et al.*, 2013) and the presence of genes that play a vital role in senescence (Payne & Chinnery, 2015). More recently, however, a single amalgamated theory that emphasizes gene repair and maintenance systems, and their surrounding is becoming more and more accepted (Rattan, 2006), stressing upon the need for a holistic, cumulative and logical analysis of senescence. However, massive research integration on aging and aging-related processes should be able to address it systematically and simplify theories on aging. More so, as commonly found by many researchers around the globe, one mechanism that is thought to contribute to this age-associated degeneration is the production of free radicals (Cui *et al.*, 2012; Sanz, 2016; Stepien *et al.*, 2017). Reactive oxygen species (ROS) are generated at multiple compartments within the cell and as accidental byproducts of multiple enzymes. Significant contributions include proteins that are localized on the plasma membrane, such as a family of oxidases, as well as the activity of various cytosolic enzymes such as cyclo-oxygenase. Peroxisomal lipid metabolism results in ROS release too. Although all these sources add up to the overall oxidative load, mitochondria still occupy the central position producing approximately 90% of total cellular ROS.

Lipid peroxidation (LPO) alludes to structural breakdown of lipids due to oxidative damage. Lipid peroxides are volatile and unstable, decomposing shortly into lipid hydroperoxides as a consequence of oxidative stress. Assaying the LPO reaction results in intermediate products like PUFA-derived stable aldehydes such as 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA) and acrolein (Ayala *et al.*, 2014). Measuring malondialdehyde has indicated the formation of covalent adducts with lipids (Lykkesfeldt, 2007), DNA (Kadiiska *et al.*, 2005), matrix proteins and inner membrane bound proteins as well (Pizzimenti *et al.*, 2013). Hence, LPO involved in loss of membrane bound proteins resulting in loss of structural integrity of the membrane (Ademowo *et al.*, 2017; Pratic, 2002; Rikans & Hornbrook, 1997). On the contrary, unsaturated fatty acids show high resistance to peroxidation in mitochondria (Gutiérrez *et al.*, 2006) and molecules like hydroperoxides generated cholesterol are more stable as well. Alteration in lipid desaturation processes culminate in reduced production of omega 3 fatty acids, which is regarded as proinflammatory in ageing condition (Flaquer *et al.*, 2015).

An increase in free radical generation usually worsens the mitochondrial physiology, partly via lipid peroxidation, respiratory chain protein oxidation, and mitochondrial DNA damage. Additionally, damaged mitochondrial membranes would shift the cellular metabolism towards glycolysis signifying the tight interplay between bioenergetics, metabolism and aging (Silva *et al.*, 2013).

Ca^{2+} in mitochondria play a vital role in accentuating the tri-carboxylic acid cycle (Griffiths & Rutter, 2009) and oxidative phosphorylation to generate ATP (Glancy *et al.*, 2013). Specifically, Ca^{2+} potentiates enzymes like pyruvate dehydrogenase, isocitrate dehydrogenase, oxoglutarate dehydrogenase, and the ATP synthase (complex V) (Das & Harris, 1990), and the adenine nucleotide translocase as well. Hence, Ca^{2+} might play a role in the increased production of ROS by augmenting metabolism. During increased metabolism, more electrons tend to escape from the ETC complexes while more O_2 gets used to synthesize ATP. To this end, basal metabolic rate and mitochondrial ROS generation have shown a positive correlation (Ku *et al.*, 1993). However, the effects of Ca^{2+} on antioxidant activities were never measured to understand the oxidant and antioxidant homeostasis in the cell. Moreover, Ca^{2+} as a cofactor in various enzymatic reactions of the cell, when sequestered by mitochondria, might skew the activities of the antioxidant indices of the cell and mitochondria in particular. Our study aims to decipher the differential role of Ca^{2+} in both augmenting and diminishing the antioxidant capacity of mitochondria. More LPO activity levels are indicators of unattended ROS levels present in the cell, signifying their activity in ageing and age related neurodegenerative diseases like stroke and

multiple sclerosis.

MATERIALS AND METHODS

MATERIALS

Ethylene glycol-bis-(-aminoethyl ether) N, N, N1, N1 tetraacetic acid (EGTA), calcium chloride, sodium succinate, N-2-hydroxy ethyl piperazine-n-2-ethane sulfonic acid (HEPES), and dimethyl sulfoxide and epinephrine were obtained from Sigma Chemicals. 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 hrs dark/light, 28±2^{o}C). Animals were let free to feed on standard food (Amruth Feeds, India) and potable water ad libitum. Animals were maintained following 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:

Mitochondria were isolated from the whole brain of Sprague Dawley rats by conventional differential centrifugation with minor modifications(Rosenthal, Hamud, Fiskum, Varghese, & Sharpe, 1987). After removal, brains were placed in isolation media which contained - 75 mm Sucrose, 20 mm HEPES buffer (pH adjusted to 7.2 with KOH), 0.1% fatty acid-free Bovine Serum Albumin (BSA) and 1 mM tetra potassium EDTA. The tissue is minced and grounded to form homogenate, which is mixed in a 1:5 ratio to buffer. After centrifugation at 11,200 g, the first pellet (nuclear fraction) is removed and the supernatant collected is layered over 1.2 M Sucrose gradient at 16,200 g for 15 minutes. Mitochondria collected as the pellet is treated with storage buffer, which is as similar to isolation buffer except having 0.1mM EDTA and is free from BSA. The solution is washed with storage buffer and spun at 1,680g for 10 minutes. Mitochondria collected are stored at -70^{0}C till further use. All the isolation procedures were carried out at 0 - 4^{0}C.

The protein concentration was determined by the Folin-Lowry method. The total protein concentration made up to 1mg/ ml in storage buffer.

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(Birch-Machin & Turnbull, 2001). All three age groups of SDH activities were measured for statistically significant results to indicate their viability (data not shown).

Lipid Peroxidation assay

Lipid peroxidation (LPO) is an important oxidative stress membrane marker assayed spectrophotometrically (Niehaus & Samuelsson, 1968) with slight modifications. The reaction involves the formation of Malanodialdehyde (MDA) at the end of lipid peroxidation activity, which readily reacts with thiobarbituric acid derivative (TBA). One mole of MDA is known to react with two moles of TBA to form a pink coloured and condensed trimethine product and the absorbance of which is measured at 535 nm.

Briefly, 100ug of mitochondria is treated with varying concentration of Ca^{2+} (0 to 300uM) along with 2ml of TBA: TCA: HCL reagent in 1:1:1 ratio of 0.37% Thiobarbituric acid, 15% Trichloro-acetic acid and 0.25N Hydrochloric acid; placed on a water bath for 10 minutes, later cooled down and spun at 1000 rpm for another 10 minutes. An aliquot of the solution measured at 535 nm with an extinction coefficient of MDA being $1.56x10^{5}$ m^{-1}/cm^{-1}, was used for calculating LPO activity. LPO activity is measured with and without Ca^{2+} to delineate the role of Ca^{2+} in the ageing brain. The values were expressed in nmoles of MDA/ g wet weight of tissue.

RESULTS

Lipids, such as cholesterol, glycolipids, polyunsaturated fatty acids and phospholipids, are important biomolecules of the mitochondrial membrane architecture. The Ca^{2+} induced mitochondrial ROS production can damage the double bonded C-C(s) of these lipids, leading to membrane lipid oxidation and thus damaging the mitochondrial form and function (Fernández-Moriano, González-Burgos, & Gómez-Serranillos, 2017). Our study indicates a slight increase of 14.5% basal LPO activity in the mitochondria isolated from aged rats when compared to neonatal rats. Similarly, 7.9% rise is evident in the young adult group compared to mitochondria isolated from neonatal rats ($p<0.001$). On considering LPO activities with Ca^{2+} concentrations of 100 uM, aged rats showed the highest activity of ~12% rise, neonatal rats showing the lowest of 6% rise, while young adults elicited 9% peroxidation activity. Increased Ca^{2+} concentration upto 300uM further increased the lipid peroxidation linearly doubing the damage activity caused by peroxidation (Fig 1 and 2). However, we conclude that Ca^{2+} has minimal role in eliciting the activity of LPO in all three age groups, when compared to the magnitude of alteration in mitochondrial membrane or ROS produced upon Ca^{2+} treatment (Turrens, 1997).

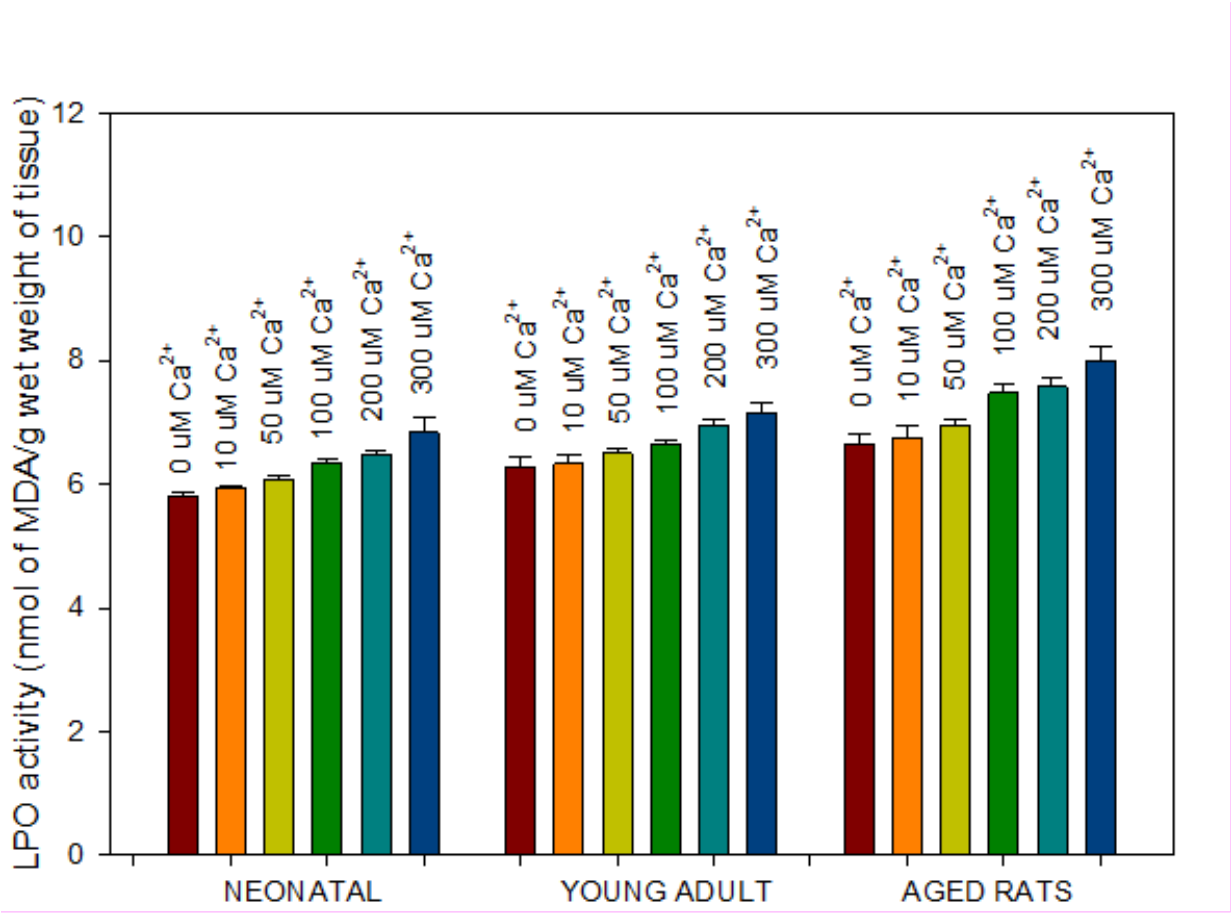
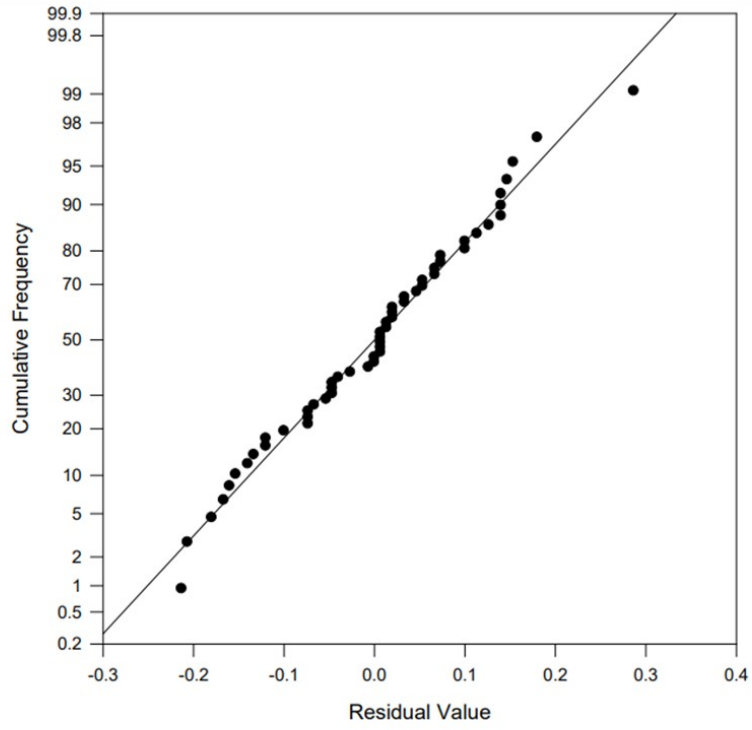


Figure 1. Effect of Ca^{2+} on LPO activity of mitochondria isolated from brain tissue of three different age groups viz., neonatal, young adult and aged rats. Mitochondria (100 µg/ml protein) isolated from neonatal rats showed low basal rate of LPO activity in control studies and gradient increase in activity with each treatment of rise in Ca^{2+} concentration. Data points represent the Mean ± SD of 5 experiments ($p<0.001$).



Source of Variation	DF	SS	MS	F	P
AGE	2	8.654	4.327	257.565	<0.001
CAL CONC	5	7.668	1.534	91.289	<0.001
AGE x CAL CONC	10	0.292	0.0292	1.741	0.01
Residual	36	0.605	0.0168		
Total	53	17.220	0.325		

Figure 2. Normal probability plot, illustrating the linear and normal frequency distribution of LPO products formed values to Ca^{2+} treatment in all three age groups. The table represents the results of Two-Way Analysis of Variance with a significant p=0.01.

DISCUSSION

Lipids play a massive role in mitochondrial double membrane architecture. Over 70% mitochondrial lipids are produced and assembled in the ER, and then sent to the mitochondria, while few such as cardiolipin and phosphatidyl-ethanolamine are produced within the mitochondria. These lipids are important in cristae assembly, its architecture and localization of proteins, enzymes, channels, pores and complexes on the inner mitochondrial membrane (Schlame & Greenberg, 2017). Our study on LPO has shown a decent rise in basal activity (without Ca^{2+} treatment) in all three age groups. Peroxidized lipids are less apolar and are known to upset mitochondrial membranes affecting the organell's overall efficiency. They do so by disrupting the membrane architecture and fluidity. Peroxidation of phospholipid products include lipid-oxides and peroxides, are major part of cellular metabolism, signaling and cell survival. Lipids like PUFA and cholesterol face oxidation at differential rates initiated by free radicals like peroxyl radical (ROO^{-}), peroxynitrite ($ONOO^{-}$), nitric oxide (NO), superoxide (O^{2-}), nitrogen dioxide (NO_2), and/or hydroxyl radicals (OH^{-}). Our result are in agreement with increased LPO in aged rats exhibiting altered mitochondrial morphology (Savitha & Panneerselvam, 2006). However, Savitha and Panneerselvam also reported the efficacy of L-carnitine and DL α lipoic acid in improving the stability of membrane structure.

In our extended study, including the role of Ca^{2+} points out at the gentle rise in rate of LPO indicating the increased ROS production. The LPO rate, though dismal, is quantitative enough to measure the deleterious effects of ROS, suggesting a direct

relationship between ROS and LPO. Lipid peroxidation products are highly reactive and include hydroxyl acids or reactive aldehydes (Gaschler & Stockwell, 2017). Assaying the LPO reaction results in intermediate products like PUFA-derived stable aldehydes such as 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA) and acrolein (Ayala *et al.*, 2014). Our experiment measuring malondialdehyde indicate the formation of covalent adducts with lipids (Lykkesfeldt, 2007), DNA (Kadiiska *et al.*, 2005), matrix proteins and inner membrane bound proteins as well (Pizzimenti *et al.*, 2013). Hence, LPO involved in loss of membrane bound proteins resulting in loss of structural integrity of the membrane (Ademowo *et al.*, 2017; Pratic, 2002; Rikans & Hornbrook, 1997). Ca^{2+} sequestration is known to vary the redox state, ionic imbalances and skew membrane potential as well. Mitochondrial altered membrane potential has a direct implication in superoxide generation and oxidant-antioxidant imbalance suggesting its contribution towards aging.

CONCLUSION

We studied the effect of Ca^{2+} on LPO induction in mitochondria isolated from brain tissue of all three age groups viz., neonatal, young adult and aged rats. Aged rats exhibiting an average of ~12% rise in lipid peroxidation to 100 µM Ca^{2+} is indicative of increased sensitivity to Ca^{2+} interplay in LPO reaction. Our results are in agreement with Ca^{2+} actively potentiating LPO (Jain & Shohet, 1981). An application of 300 µM Ca^{2+} further doubled the rate of lipid peroxidation in aged rats to 20%, which should be significantly altering the phospholipid content in the mitochondrial inner membrane, compromising the membrane stability and architecture. In summary, age related free radical production and accumulation has been suggested to be harmful to major components of the cell and would promulgate aging and age-related diseases like Alzheimer's disease (Simoncini *et al.*, 2015). The decrease in LPO activity as seen in young adult rats may be due to stable and healthy adult life, as seen in all animals.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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