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Differential effect of Ca^{2+} on mitochondrial superoxide dismutase and catalase activities in the aging brain

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Abstract
Mitochondrial participation in cellular free radical generation constitutes more than 80% of the total. Free radicals tend to be harmful and cause oxidation of proteins and lipids in the cell and hence they are proposed as an important factor affecting aging. However, the antioxidant capacity to catapult oxidative stress in mitochondria is imminent to delay aging. More so, mitochondrial Ca^{2+} sequestration has some profound effect on ROS production and detoxification. In the current study, we have investigated the effect of Ca^{2+} on age-related alterations in antioxidant enzymes like sodium dismutase and catalase activities. The spectro-photometric measurement of both sodium dismutase and catalase has shown a differential response to Ca^{2+}. Sodium dismutase has shown a downward trend in activity for varying concentrations of Ca^{2+} treatment in aging while catalase has not shown much deviation in its activity levels, except for a small rise in aged animals. Thus, Ca^{2+} seems to be differentially affecting the activity of two antioxidant enzymes in the mitochondrial isolate.

Keywords: aging; SOD; catalase; antioxidant; Ca^{2+}

Introduction

Aging has been ascribed to changes at molecular intersections and impairments due to cellular free radical generation [1], alterations in immunity [2, 3], telomere shortening [4]. and the presence of genes that play a vital role in senescence [5]. Reactive oxygen species produced during routine cellular metabolism of substrates in multiple compartments of cells are to be blamed for the age-related damage and diseases at the cellular and tissue levels. Normal healthy cells exhibit oxidants, antioxidants, and biomolecules in a well-balanced state of affairs. However, surplus production of ROS may overpower natural cellular antioxidant defenses to prevent cellular functional repair pathways. 100% pure oxygen (O_2) consumed at 1 atmosphere would kill a rat within 3 days. However, rats exposed to 80% O_2 for 5 days, learn to adapt and survive in 100% O_2 till they achieve a natural death. O_2 is a double-edged sword sustaining life and having the ability to take away the same too. For instance, doctors early in the 1940s utilized 100% O_2 to sustain life in infants without realizing 21 % O_2 was adequate to treat "retinopathy of prematurity" in newborn babies. Later, the application of lipid-soluble antioxidant α-tocopherol to babies was identified to reduce the incidence of retinopathy [6]. In another instance, in the early 1970s, researchers found that increased SOD activity in the lung elevated chances of rat's survival to hyperoxia [7].

O_2 is being utilized to produce metabolic water in mitochondria. Thus, O_2 if effectively not loaded with 4H+, then there is an occasional chance of electron leakage from the electron transport chain (ETC). These leaked electrons knock O_2 to form superoxides, a progenitor of free radicals. Free radicals interact indiscriminately injuring almost all types of cellular components. To combat these deleterious effects of free radicals, cells have evolved a battery of enzymes in their stock to neutralize oxidants. These broad ranges of antioxidants, existing both endogenously and exogenously, are always at disposal to guard the cellular components from the effects of the oxidative load.

The superoxide dismutases (SOD) catalyze two superoxide anions to hydrogen peroxide and oxygen molecules using cyclic redox reactions. The redox shuffling of two protons per cycle at the metal present in the active center performs the catalytic reaction called dismutation [8].

$Mn^{3+} + O_2^- \leftrightarrow Mn^{2+} + O_2$

$Mn^{2+} + O_2^- + 2H^+ \leftrightarrow Mn^{3+} + H_2O_2$

Overall reaction: $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

The hydrogen peroxide produced must then be detoxified by catalases or glutathione peroxidases in separate pathways (will be discussed below). SOD exists in three forms in mammalian tissues, each with a specific metal ion at the active center, subcellular location, and distribution.

(1) Copper zinc superoxide dismutase (Cu-Zn SOD): Cu-Zn SOD has MW of 32,000 kDa and is found in the cytoplasm and organelles as well.

(2) Manganese superoxide dismutase (Mn-SOD): Mn-SOD has an MW of 40,000 kDa and is specific to mitochondria in distribution[9]. Mn-SOD has 4 protein subunits, each probably holding an Mn^{2+} atom. Mn-SOD and Cu-Zn SOD have their dissimilarity tracked at amino acid sequence and inhibition by cyanide is not specific to the latter.

(3) Extracellular superoxide dismutase (ECSOD): EC-SOD is produced by fibroblasts and endothelial cells only, and is localized on the cell surface where it binds to heparan sulfates.

Catalase is the first antioxidant enzyme to be characterized. It has 4 subunits of protein and each of these contains a haem group and a molecule of NADPH at the center [10]. It is known to convert hydrogen peroxide to water and oxygen in two-step reactions:

CAT – Fe (III) + $H_2O_2 \rightarrow$ Compound I

Compound I + $H_2O_2 \rightarrow$ CAT–Fe (III) + $2H_2O+O_2$

The above reaction follows a high rate constant of ~107 M/sec, signifying the untiring catalase enzymes activity *in vivo*. Peroxisomes contain a large volume of catalases, though other

organelles have their share of the enzyme too. Mitochondrial catalase has a huge role in detoxifying free radicals that are routinely produced. However, catalase quantification and distribution among organelles are ambiguous as methods involved to isolate organelles can easily disrupt peroxisomes and release catalases onto the isolate. Liver and RBCs have the greatest amount of catalase activity. However, the brain tissue does show a quantifiable amount of catalase activity.

Ca^{2+} in mitochondria play a vital role in accentuating the tri-carboxylic acid cycle [11]. and oxidative phosphorylation to generate ATP [12]. Specifically, Ca^{2+} potentiates enzymes like pyruvate dehydrogenase, isocitrate dehydrogenase, oxoglutarate dehydrogenase, and the ATP synthase (complex V) [13], 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 [14]. 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. Both SOD and catalase activity levels are indicators of unattended ROS levels present in the cell, signifying their activity in aging and age related neurodegenerative diseases like stroke and multiple sclerosis.

2. Materials and Methods

2.1 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±2ºC). 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.

3. Methods

3.1 Isolation of Mitochondria

Mitochondria were isolated from the whole brain of *Sprague Dawley* rats by conventional differential centrifugation with minor modifications [15]. 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.

3.2 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 [16]. All three age groups of SDH activities were measured for statistically significant results to indicate their viability (data not shown).

3.3 Superoxide dismutase assay

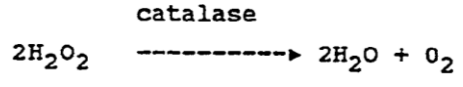
Superoxide dismutase (SOD) activity is a measure of inhibition of auto-oxidation of epinephrine. The superoxide anion generated in the mitochondria causes the oxidation of epinephrine to adrenochrome and adrenochrome, in turn, churns out more free superoxide radicals, accounting for an unregulated chain reaction. Additionally, the increase in pH from 7.8 to 10.2 augments the rate and sensitivity of autooxidation of epinephrine thus aiding in SOD measurement.

To 100 µg of a mitochondrial sample, the varying concentration of Ca^{2+} *viz* 0 for control, and up to 300 µg Ca^{2+} was added individually. To the Ca^{2+} treated sample, 60uL of epinephrine (30mM in 0.05% acetic acid) was added to initiate the reaction. The resulting absorbance was recorded at an interval of 30s for 4 min at 480 ηm. One unit of SOD is defined as the amount of enzyme present in the sample to inhibit the autoxidation of epinephrine by 50%. SOD and its activity are expressed in ηmols/min/mg of protein.

Note: Mitochondria were isolated afresh in a phosphate buffer containing no EDTA or EGTA, as they chelate Ca^{2+} ions.

3.4 Catalase assay

Catalase is a ubiquitous enzyme found in all aerobes, known to reduce 2 molecules of hydrogen peroxide to molecular oxygen and two molecules of water. Under ultraviolet (UV) illumination, catalase would continue to act on hydrogen peroxide decreasing its absorbance values at 240 ηm and facilitating its measurement.



The reaction mixture containing 100g of mitochondrial sample extracted in phosphate buffer (pH 7.0) was taken along with the

varying concentration of Ca^{2+} *viz* 0 for control, up to 300 µg Ca^{2+} in different gradations individually for each set of experiment. The reaction was initiated by the addition of 2mM H_2O_2 at 4^0 C. The decrease in absorbance at 240 ηm was followed for 3 minutes at 30 seconds interval. Catalase activity is calculated using the formula

$K= (2.303/t) X (\log A_1/\log A_2)$

Where A_1 and A_2 are the absorbance values recorded at a time interval, t in seconds and K is the rate constant. The values are expressed in µmoles of H_2O_2 hydrolyzed/min/mg protein. Specific activity expressed as units/mg protein.

4. Result

Superoxide radicals stem from accidental electron leakage of the mitochondrial respiratory chain complexes while metabolizing simpler units of biomolecules; resulting in oxygen toxicity. This process gives rise to the generation of singlet oxygen and hydroxyl radicals. These two are known to be the progenitors of all free radicals generated in the cell and hence can be recognized as two of the most powerful oxidants known. These free radicals generated in various compartments of mitochondria are known to oxidize cellular components indiscriminately and deteriorate them. To counter these free radicals, specific Mn-Superoxide dismutases (SOD) are produced in the mitochondria as well. SODs are known to dismutate superoxide into oxygen and H_2O_2, which are less harmless products. We measured the SOD activity in isolated mitochondria of all three age groups *viz*., neonatal, young adult, and aged rats. Our study indicates a declining activity of SOD in all three age groups.

In the current investigation, we found that mitochondria isolated from young adult rats showed a negligible amount (<1%) of SOD activity in comparison to neonates. However, mitochondria isolated from aged rats showed a drastic decrease of ~17% in SOD activity (Fig.1).

Ca^{2+} sequestered in mitochondria is known to alter several key functions of mitochondria like ATP production, apoptosis, fatty acid metabolism, and reactive oxygen production and detoxification. The role of Ca^{2+} is investigated further in the SOD activity of all three different age groups. Ca^{2+} has shown to affect SOD activity considerably. Ca^{2+} addition, in different concentrations *viz* 0, 10,50,100,200 and 300ug has decreased the SOD activity in a linear fashion. Our study pointed out a decrease of ~18%, ~10%, and 38% SOD activity in comparison to mitochondria isolated from neonatal, young adult and aged rats upon 100uM Ca^{2+} treatment (Table 1). However, 300uM Ca^{2+} treatment to the mitochondria isolated from all the age groups proved to be detrimental in the proportion of up to ~39-44% loss in SOD activity. However, mitochondria isolated from young adult rats have shown a slight resistance to the harmful effect of Ca^{2+}.

In the current investigation, we found that mitochondria isolated from young adult rats showed a negligible amount (<1%) of catalase activity in comparison to neonates (Fig.2). However, mitochondria isolated from aged rats showed a drastic increase of ~12% in catalase activity without any external treatment of Ca^{2+}. Mitochondrial Ca^{2+} sequestrations in the brain tissue affect several physiological phenomena within and outside mitochondria, for example, Ca^{2+} extrusion amplifies synaptic plasticity between two neurons. Excessive Ca^{2+} within mitochondria can denature the mitochondrial membrane releasing Cytochrome-c and several other factors participating in apoptosis. Thus, it is pertinent to understand the effect of Ca^{2+} on antioxidant enzymes like catalase to combat oxidants generated within the mitochondria. Ca^{2+} has affected several antioxidants in the mitochondria but catalase seems to be resistant to it. Treatment of Ca^{2+} from 0-300uM resulted in a mere 1% rise in catalase activity in both neonatal and young adult rats (Table 2). However, aged rats have shown a 6% rise in its activity to the maximum load of Ca^{2+} in our experimental setup.

5. Discussion

In 1969, Dr. Irwin Fridovich discovered Superoxide dismutase (SOD) to be scavenging on superoxide radicals to dismutate them into oxygen and hydrogen peroxide molecules [17]. Since then, an enormous amount of research has been done on SOD and researchers have identified 3 forms of eukaryotic SOD having different metal ions in their catalytic center thus exhibiting variation in their efficacy. SOD has been identified to be ubiquitously existing in all organisms on earth and, thus, is essential for the existence of life.

MnSOD is specifically localized in the mitochondrial matrix where the highest rate of superoxides is generated due to electron leakage from ETC. Superoxides are the result of the one-electron reduction of diatomic oxygen to form superoxide [18]. MnSOD's are the only scavengers to act directly on superoxide levels in the mitochondria and convert them into harmless products and hence are associated with increased lifespan in eukaryotes [19]. The mutational study involving MnSOD knockout mice, the mortality rate increased within ten days of life. Loss of life in mice is due to dilated cardiomyopathy, fatty liver, and metabolic acidosis [20]. However, MnSOD overexpression in fruit flies is found to increase the lifespan of the flies [21]. Alternatively, 100% oxygen at 1 atm exposure is known to kill rats due to hyperoxia, pulmonary toxicity, and oxidative stress. However, rats exposed to 21% to 100% oxygen in gradation for 3 days, were able to adapt to combat hyperoxia, as they showed increased SOD activity in the lungs [7, 22].

The current study employing the SOD activity measurement in mitochondria isolated from three different age groups found SOD activity to be decreasing as a function of age. The negligible improvement of 1% rise we see in young adult rats is due to the health and fitness of the organism. This correlates directly to the incompetence of the mitochondria to quench free radicals to harmless products with increasing age. Oxidative stress is one of the primary causes of aging and age-related pathologies including AD, PD, stroke, and others [23]. The imbalance created between oxidants and antioxidants in brain tissue leads to neurodegeneration [24]. More so, a study on Drosophila has revealed SOD activity to be directly proportional to natural aging [25]. To support further, Kevin L, Quick, *et al*., studied SOD mimetics like carboxy fullerenes which improved memory deficits and lifespan in mice [26]. Studies on extracellular SOD has corroborated attenuating age-dependent cognitive deficits, suggesting a newer target for drug development [27].

Ca^{2+} sequestration alters several parameters in mitochondrial physiology that if it gets skewed, would be disastrous to the cell. In neurons, Ca^{2+} excitotoxicity is known to be the primary reason behind the stroke. Our results suggest that Ca^{2+} has a detrimental role in SOD activity. With each graded addition of Ca^{2+} to the isolated mitochondria from the brain tissue of three different age

groups, there was a decline in SOD activity. The reason for this action is quite elusive. Ca^{2+} aggravates the free radical production in mitochondria. Superoxide thus produced tends to form permeability transition pore and form a gateway for apoptosis [28]. Our study is indicative of an inverse relation with SOD action and ROS production. This may be due to two reasons – one, SOD activity is hindered directly by the presence of Ca^{2+} and secondly, SOD's are being denatured upon Ca^{2+} uptake by the mitochondria. Either way, SOD activity measurement in all the three age groups suggests the declining antioxidant capacity of the enzyme while aggravating the oxidant force to mitochondrial dysfunction.

All living organisms express catalase to combat free radicals. Catalase exhibits the highest turnover rate among all enzymes; a single molecule of catalase can safely convert around millions of H_2O_2 molecules to H_2O and O_2 each second [29]. Overexpression of catalase and/or its external mimetics treatment is known to extend life and healthspan in murine and hence many believe that catalase activity is longevity determinant enzyme [30, 31]. Our study targeting catalase activity in relation to aging found a 13% rise in activity indicating the elevated levels of the antioxidant produced is to lower the burden of free radicals produced in the aged rats. Brain tissue has restrained itself in effectively producing enhanced catalase activity to protect the cell from oxidative damage, compared to other tissues like the liver [32]. However, increased catalase activity is known to delay premature aging in long-lived Ames dwarf mice [33]. Our study is in agreement with several studies identifying elevated levels of catalase activity in the aging tissue [34, 35].

The calcium intervention does not seem to raise any significant rise in catalase activity. It seems catalase may be active independent of Ca^{2+} levels in the mitochondria, although a slight rise of 6% was observed in aged animals upon 100 to 300µM Ca^{2+} treatment. Physiologically, mitochondrial Ca^{2+} loading would not be this high and hence it cannot be correlated to its effect as the study has recorded.

6. Conclusion

In summary, Ca^{2+} disrupts antioxidant activities of mitochondrial SOD and catalase in the brain tissue. The deranged Ca^{2+} homeostasis in mitochondria is the root cause of many neurodegenerative diseases like stroke, multiple sclerosis, Alzheimer's and Parkinson's, and others. Thus, the current investigation on the activity measurement of the two of the most commonly found antioxidants like SOD and catalase has shown a differential effect for Ca^{2+} induction. SOD followed a decreasing trend in activity levels to the increasing concentration of Ca^{2+} while catalase maintained unchanged activity levels in both neonates and young adult rats. However, aged rats showed a small yet significant rise of 6% in catalase activity levels. However, a better comprehension of the factors controlling the antioxidant stability and activity requires a holistic approach and ROS interplay to address the molecular mechanisms underlying aging and neurodegenerative pathologies.

7. Authors Statement

Both authors equally involved in the execution of experimental work, and preparation of the manuscript and have no competing and conflict interests to declare.

8. Acknowledgment

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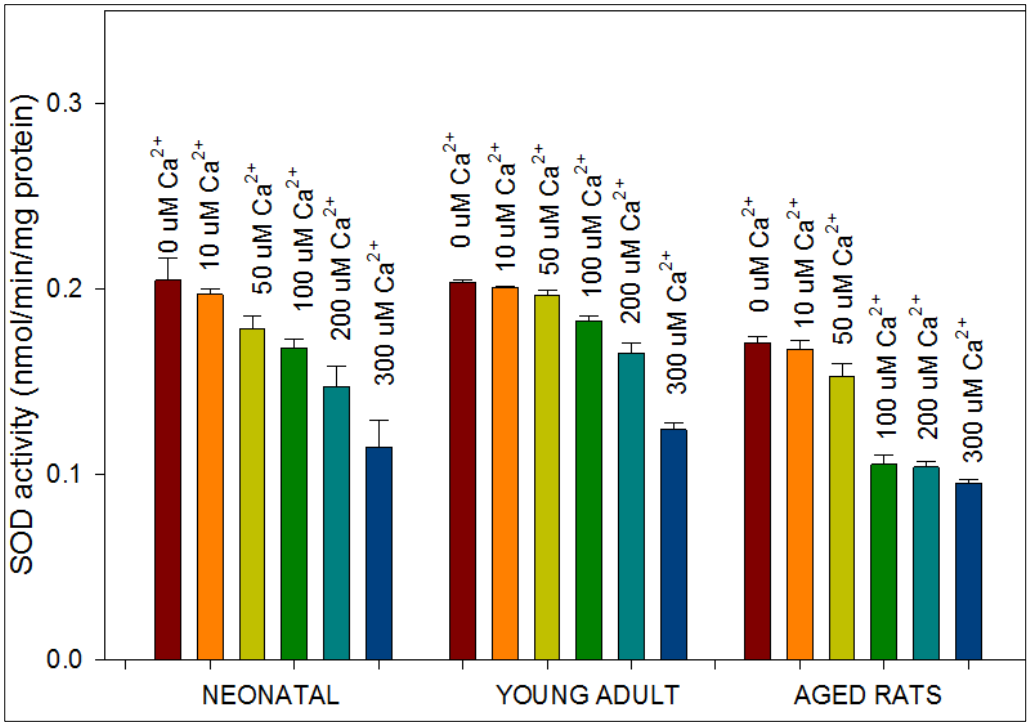

Fig 1: Effect of Ca^{2+} on SOD activity of mitochondria isolated from brain tissue of three different age groups *viz*., neonatal, young adult and aged rats. Mitochondria (100 µg/ml protein) isolated from aged rats showed a low basal rate of SOD activity in control studies. Data points represent the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

Table 1: The table indicates the isolated mitochondria being treated with various concentrations of Ca^{2+} *viz* 0 to 300 µM and SOD activity being measured in three different age groups. Values are Mean ± SD of 5 replicates represented as the mg/g protein. 300uM Ca^{2+} treatment has shown a maximum decline of 44% activity in aged and neonatal rats. Mitochondria isolated from young adult and neonatal rats have shown a similar trend of activity decline with each rise in Ca^{2+} treatment.

AGE/SOD activity	0 µM Ca^{2+}	10µM Ca^{2+}	50µM Ca^{2+}	100 µM Ca^{2+}	200µM Ca^{2+}	300µM Ca^{2+}
NEONATAL	0.2045±0.012	0.1971±0.003	0.1787±0.006	0.1682±0.005	0.1474±0.011	0.1147±0.014
Percentage of activity change		-3.90	-12.68	-18.05	-28.29	-43.90
YOUNG ADULT	0.2036±0.002	0.2007±0.001	0.2017±0.002	0.2023±0.003	0.1652±0.005	0.1243±0.003
Percentage of activity change	-0.49	-1.47	-3.92	-10.29	-19.12	-39.22
AGED RATS	0.1708±0.004	0.1674±0.005	0.1531±0.007	0.1055±0.005	0.1039±0.003	0.0956±0.002
Percentage of activity change	-16.59	-2.34	-10.53	-38.01	-39.18	-43.86

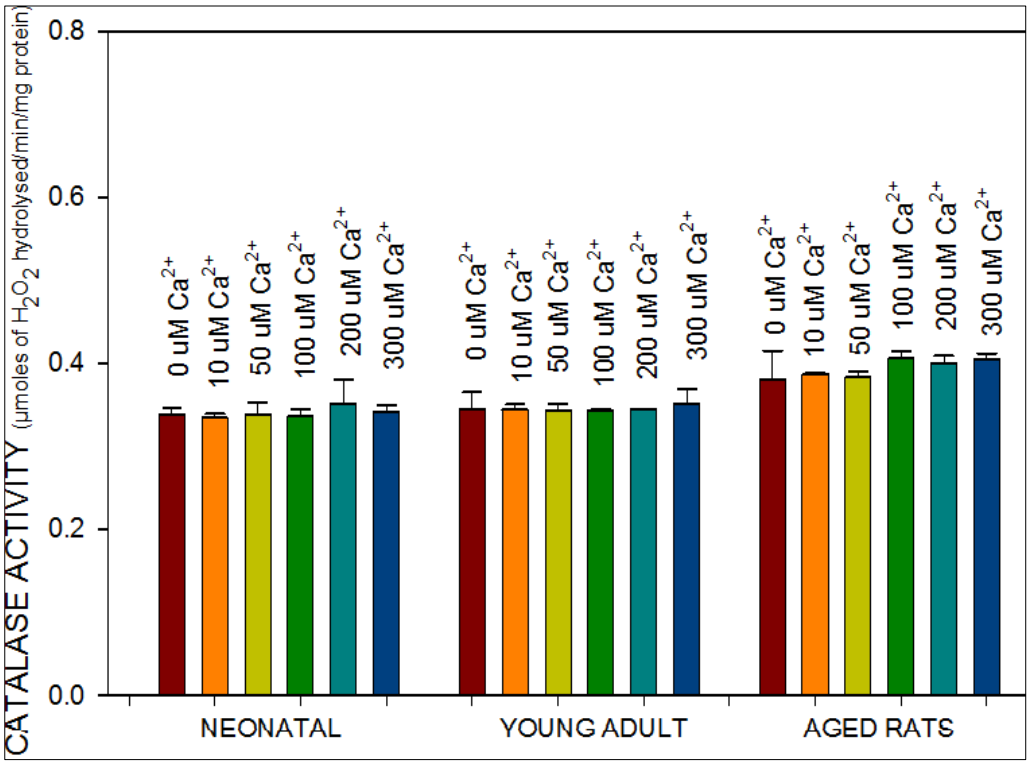

Fig 2: Effect of Ca^{2+} on Catalase activity of mitochondria isolated from brain tissue of three different age groups *viz*., neonatal, young adult and aged rats. Mitochondria (100 µg/ml protein) isolated from aged rats showed a low basal rate of catalase activity in control studies. Data points represent the Mean ± SD of 5 experiments ($p<0.05$).

Table 2: The table indicates the isolated mitochondria being treated with various concentrations of Ca^{2+} *viz* 0 to 300 µM and SOD activity being measured in three different age groups. 300uM Ca^{2+} treatment has shown a maximum decline of 6% activity in aged and neonatal rats. Mitochondria isolated from young adult and neonatal rats have shown a similar trend of activity decline with each rise in Ca^{2+} treatment. Values are Mean ± SD of 5 replicates represented as the mg/g protein.

AGE/Catalase activity	0 µM Ca^{2+}	10µM Ca^{2+}	50µM Ca^{2+}	100 µM Ca^{2+}	200µM Ca^{2+}	300µM Ca^{2+}
Neonatal	0.338±0.008	0.334±0.005	0.339±0.014	0.337±0.008	0.352±0.029	0.342±0.007
Percentage of activity change		-1.18	0.30	-0.30	4.14	1.18
Young adult	0.345	0.344	0.343	0.344	0.344	0.347
Percentage of activity change	2.071006	-0.29	-0.58	-0.29	-0.29	0.58
Aged rats	0.381±0.034	0.387±0.015	0.384±0.069	0.406±0.089	0.4±0.086	0.404±0.078
Percentage of activity change	12.72189	1.57	0.79	6.56	4.99	6.04

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