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Melatonin Modulates Neuronal Mitochondria Function During Normal Ageing in Mice

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Summary: Mitochondrial dysfunction has been shown to be associated with normal ageing and may account for age-related vulnerability to disease. The increasing number of old people worldwide has created the need to find effective therapeutic agents to reduce the incidence of age-related disease. In the current report, we carried out an assessment of mitochondrial function in established young, middle-aged and old synaptosomal mitochondria bearing cybrids without or with melatonin treatment. The cybrids were generated by transferring isolated mitochondria from synaptosomes of brain cortical cells in mice to rho-zero mtDNA-less cells. In galactose media, a selective media that tests a cells ability to produce ATP through the electron transport chain and oxidative phosphorylation, 500µM melatonin (N-acetyl-5-methoxytryptamine) raised cell viability in young and middle-aged cybrids ($P<0.05$) and a concentration of 1mM raised cell viability in the old cybrids ($P<0.05$). The mitochondrial membrane potential (MMP) was lowered in the young cybrids ($P<0.05$) treated with melatonin, but it was raised in the middle-aged and old cybrids ($P<0.05$) with melatonin treatment. The levels of reactive oxygen species were significantly lower in the melatonin treated middle-aged and old cybrids compared with controls ($P<0.05$). Furthermore, ATP measurements showed no significant increase in the young cybrids ($P>0.05$), but increased significantly in the middle-aged and old cybrids ($P<0.05$) with melatonin treatment. Light and fluorescence microscopy showed observable structural damage and cell death in the middle-aged and old cybrids without melatonin treatment. The results suggest that melatonin may be a potent therapeutic intervention during age-related neuronal mitochondrial dysfunction.

Keywords*:* Melatonin; Synaptosomes; Neuronal mitochondria; Ageing

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INTRODUCTION

Ageing is a series of post-maturational processes leading to diminished homeostasis and an increased vulnerability to age related disease (Masoro, 1995; Troen, 2003). In 2012, the world population of persons 60 years or over was approximately 810 million and is expected to grow to about 2 billion by 2050 (United Nations, 2012). The worlds' ageing population is projected to increase by more than 50% in the next three decades (United Nations, 2012). Often associated with ageing are the age-related dysfunction and degenerative diseases that have challenged researchers to search for a solution to improve the quality of life in the elderly. The need to identify an effective therapy to preserve homeostasis has been intensified with the growing ageing population. Furthermore, given that the health challenges in the elderly are diverse and pleiotropic, it is needful to identify an effective therapeutic target that will reduce the susceptibility of the ageing population to the different systems diseases.

The proper functioning of the mitochondria bioenergetics machinery is important for overall normal cellular function. Earlier reports in cell models of ageing support that mitochondria DNA loss and consequently a defective electron transport chain and oxidative phosphorylation are present during ageing (Miquel et al., 1980; Miquel et al., 1992; Hiona and Leeuwengurgh, 2008; Li et al., 2010). Furthermore, altered cellular function observed in ageing and age-related disease show involvement of mitochondrial dysfunction (Paradies et al., 2010a; Paradies et al., 2010b., Santos et al., 2010a, Santos et al., 2010b; Wang et al., 2011; Hauser and Hastings, 2013). An earlier study on mitochondria from synaptosomes from young, middle-aged and old mice showed that there exist age-related changes in mitochondrial function (Li et al., 2013).

Melatonin has been proposed to be an anti-ageing therapy but clinical evidence is still insufficient (Luchetti et al., 2010). The signaling pathways are complex and are yet to be clearly identified and described (Dragicevic et al., 2012). Melatonin has shown to improve cell viability during oxidative stress in normal cells and at the same time inhibit the growth of cancer cells (Bejarano et al., 2009). Alterations in mitochondrial parameters present during brain ageing

were counteracted by long term melatonin administration (Akbulut et al., 2008; Paradies et al., 2010a). Among antioxidants that protect the mitochondria, melatonin has been found to be the most clinically accessible and effective target to improve mitochondria function (Wang et al., 2011; Lowes et al., 2013). The strong evidences for melatonin role in the regulation of mitochondria bioenergetics is based on; the presence of melatonin receptors on the mitochondria (Wang et al., 2011), the highest concentration of melatonin in the mitochondria compared with other cell organelles (Martin et al. 2000; Martin et al., 2002); and its strong antioxidant and free radical scavenging potential (Pieri et al., 1994; Reiter 1995; Reiter 1998). Furthermore, during ageing, melatonin levels are low (Waldhauser et al., 1988), and may account for low total antioxidant strength of the cell.

Brain tissue has a high energy demand and susceptible to free radical induced damage. Hence, ageing is often associated with memory and motor deficits in the brain (Bondy and Sharman, 2007). Nevertheless, neuroprotection has been supported with evidences such as the increased survival of neurons with melatonin treatment (Ramirerez et al., 2009). We assessed the role of melatonin on mitochondrial function during ageing in cybrids bearing mitochondria from young, middle-aged and old mice. The results point to the mitochondrial origin of age-related cellular dysfunction and the therapeutic potential of melatonin to improve mitochondrial function during normal ageing.

MATERIALS AND METHODS

Generation of cybrids bearing mitochondria from brain cells

Eighteen female C57BL/6 mice were purchased from Charles River Laboratories. The animals (6 in a group and aged, 6 months, 13 months and 26 months old) were housed at the UTHSCSA animal facility. The animals were cared for following the institutional animal care committee rules. All experimental procedures were approved by the same committee. Briefly, the brain was dissected out and synaptosomes were derived from the brains of the animals by the method described by [Polosa and Attardi, 1991 and Li et al., 2013]. Synaptosomal mitochondria bearing mtDNA were obtained from young, middle-aged and old mice. Brain cortices were dissected out from the cerebral hemispheres and rinsed with Hanks Balanced Salt solution (Invitrogen) in a 10mm dish placed on ice. This was followed by rinsing the brain samples in Medium I (10% 0.3M sucrose, 5mM TrisHCl- pH 7.5, 0.1mM EDTA - pH 7.0) and further processed with a type A Dounce homogenizer for 16 strokes. Samples were centrifuged at 1310g for 5 minutes at 4°C followed by centrifuging the supernatant at 12,000g for 5 minutes. The pellet was re-suspended in 1ml Medium (I) per 1 gram of brain tissue and diluted with 4 vol of 8.5% Percoll solution (18.1ml of Medium 2 – 0.25M sucrose, 5mM TrisHCl – pH 7.5, 0.1mM EDTA – pH 7.0) and 1.9ml of the stock solution of isosmotic Percoll (SIP; 9 Vol of Percoll solution, 1 volume of 2.5M sucrose). Following this, 2ml of the mixture was layered on to a pre-chilled Sucrose/Percoll density gradient. 2ml 16% Percoll was added to the bottom of the centrifuge tube followed by slowly adding 2ml of 10% Percoll on the 16% percoll. The gradients were centrifuged at 18,000g for 20 minutes. The synaptosomal mitochondria enclosed by a cell membrane were collected between 8.5% and 10% gradient of the total 1-2ml. The collected synaptosomal mitochondria were washed in Medium II, and centrifuged for 20 minutes at 18,000g. The generation of cybrids was carried out as described by (Li et al., 2010). Briefly, approximately 10^6 $p^{0\ cells}$ were mixed with synaptosomal mitochondria prepared from about 1g of brain tissue. The mixture was centrifuged for 10 minutes at 2,000g, and the pellet re-suspended in 45% Polyethylene Glycol (PEG) by mild and brief pipetting. The fusion was stopped after 10 minutes by adding 10 volumes of Dulbecco Modified Eagle's Medium (DMEM). The fusion product was incubated in DMEM supplemented with 50µg/ml uridine at 37^0C for 24 hours. The medium was replaced by a semi-selection medium (10µg/ml uridine) after 24 hours. After five days, the medium was changed to the same medium without uridine. Transformants were selected based on auxotrophy of p° cells as cybrids bearing exogenous mitochondria from synaptosome were able to survive in the selective medium. The mtDNA-less p° LL/2-m21 cell-line used in this fusion is a derivative of the mouse cell line LL/2 as described previously by (Bai et al., 2000). All the cybrids were grown in a monolayer culture form in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, FBS (Invitrogen).

Cell Culture and treatment

Cells were first seeded in 100mm culture dishes, approximately $5x10^5$ cells per culture dish. After about 48 hours when the cells are attached and have shown considerable growth number, they were removed, washed in PBS and re-cultured in equal numbers in each well of the culture plates. In seeding cells for the experiments, each well in the 6-well culture plate was seeded with 400-500 x 10^3 cells and each 100mm culture plate was seeded with 1 x 10^6 cells. The cybrids were grown for about 24 hours on 6 well plates, 12 well plates and 100mm culture dishes. The cybrids / cells were tested in glucose and galactose media. The growth media used are: DMEM which contains 4.5mg/ml glucose and 0.11mg/ml pyruvate and DMEM lacking glucose but contains 0.9mg/ml galactose and 0.11mg/ml pyruvate, both supplemented with 10% dialyzed Fetal Bovine Serum (FBS) and 5%

antibiotic. Melatonin was weighed and dissolved in Dimethyl sulphoxide (DMSO).

Cell Viability Assay

The cell viability assay was carried out by a method described by (Mosmann, 1983), using a color reaction as a measure of viable cell number. Tetrazolium salts measure the activity of several dehydrogenase enzymes by cleaving of the tetrazolium ring in an active mitochondria such that the reaction occurs in living cells only (Mosmann, 1983; Slater et al., 1963). 5mg of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was dissolved in 1ml of Phosphate Buffered Saline (PBS). After treatment for 48 hours, cells were detached and washed in PBS. Samples were centrifuged at 3000g for five minutes in 1.5ml eppendorf tubes. Cells were then re-suspended in 200µL growth media. 20µL MTT solution was added to 200µL sample in all the tubes of the assay and incubated for 1.5 to 5 hours at 37^0C and 5% CO_2 in a Forma Scientific Inc., CO_2 Water Jacketed Incubator Series II. Following incubation for 1.5 - 5 hours, acid-isopropanol (10% Triton X 100, 0.1N HCl in anhydrous iso-propanol - 125ml) was added to dissolve the dark blue formazan crystals resulting in a purple solution after removing growth medium. Each sample was transferred to 96 well plates and absorbance was read at room temperature using the Biotek Instruments microplate reader at a test wavelength of 570nm.

Measurement of Mitochondria Membrane Potential (MMP)

The mitochondrial membrane potential, ΔΨM, is the potential gradient generated across the inner membrane of the mitochondria as a result of transfer of electrons along the electron transport chain. This reductive transfer of electrons through the respiratory complexes I –IV results in the pumping of protons across the inner mitochondrial membrane outside the mitochondria matrix. The accumulation of protons outside the mitochondria matrix creates an electrochemical gradient which flows back into the mitochondria creating the energy required to generate ATP (Perry et al., 2011). To measure MMP, cells with active mitochondria were washed severally to remove media completely and re-suspended in homogenization buffer (PBS) with a minimum volume of 100/200µl. This mixture was centrifuged at 3,000g for 5 minutes to pellet the cells. The pellet was treated with 200nM Tetramethyl Rhodamine Methyl Ester (TMRM), Hoechst at a concentration of 10µg/ml in DMEM and incubated for 20-30 minutes at 37^0C. After incubation, the pellet was centrifuged at high speed, 12,000g and re-suspended in homogenization buffer. This step was repeated three times to remove excess TMRM and in the incubation medium. The pellet was re-suspended in the homogenization buffer with a volume of 100µl and fluorescence measured using the Biotek Instruments Microplate Reader at Excitation (Ex) 540±10nm and Emission (Em) 570±10nm. The readings were normalized using protein content and with Hoechst stain measurement, Excitation at 360nm and Emission at 465nm.

Measurement of Reactive Oxygen Species

Reactive oxygen species are free radicals generated from the oxidative phosphorylation and they have been implicated in oxidative stress and cell death processes. The Mitrotracker Red CMH2XROS, Invitrogen (molecular weight 497.08 - a derivative of X-Rosamine) was used to determine the level of reactive oxygen species generated from the cybrids according to the protocol described by Invitrogen. Briefly, the staining solution was prepared by adding 100µl Dimethyl sulphoxide (DMSO) to make 1mM stock solution and stored in -80^0C. The 1mm MitoTrackerR stock solution was diluted to final working concentration of 1µM in growth medium - Dulbecco's Modified Eagle Medium (DMEM), with or without serum to match the medium in which the cells were grown in. After melatonin administration, cells were washed in PBS, followed by centrifugation at 3000g to obtain a cell pellet and the supernatant aspirated. The cells were re-suspended gently in pre-warmed (37^0C) medium containing the MitoTrackerR (1µM) and Hoechst (10µg/ml). Following this, the cells were incubated for 15-45 minutes under growth conditions appropriate for the cells (cybrids). Centrifugation (at 12,000g) followed to pellet the cells after which the cells were washed in PBS. The washing step was carried out thrice to remove growth medium. After washing, the pelleted cells were re-suspended in 100µl PBS and transferred to 96 well black bottom plates. Fluorescence was measured in the Biotek Instrument Microplate Reader at 530/25-590/35 nm for MitoTracker Red and at 350 – 450nm for Hoechst. The data was normalized by Hoechst and protein estimation.

Measurement of ATP

Adenosine triphosphate was measured using the ATP Assay (Invitrogen) kit. 10ml of standard reaction solution was prepared in a 50ml tube placed on ice. The standard reaction solution contained; 8.9ml distilled water, 0.5ml 20X reaction buffer, 0.1ml 0.1M DDT (Dithiothretol), 0.5ml of 10ml of 10mM D-Luciferin and 2.5µl of firefly luciferase 5mg/ml stock solution. The reaction solution was protected from light and stored at 4^0C before use. Approximately 1 X 10^6 – 2 X 10^6 cells were used for each measurement. After trypsinization, the cells were washed in Phosphate Buffered Saline (PBS). The 20X lysis buffer was prepared according to the recipe given by Invitrogen: 200mM Tris (pH 7.5), 2M NaCl, 20mM EDTA, and 0.2% Triton-X-100. The washed cells were re-suspended in 1X lysis buffer followed by sonication on ice. The mixture was centrifuged at high

speed 12,000g and the supernatant taken for further analysis. 10µl was taken for ATP measurement. ATP standard reaction solution (Invitrogen) was added to each well in the microplate to make 100µl solution and ATP was assayed promptly by reading the Luminescence at 100 and or 200 sensitivity using the Biotek Instrument Miroplate reader.

Microscopy

Light and fluorescence microscopy was carried out using the Nikon Eclipse T*I m*icroscope. Cells were viewed under x100 and X400 magnification.

Statistical Analysis

Data is presented as mean ± standard error of mean (SEM). Statistical analysis was carried out on data using the unpaired Student's *t*-test.

RESULTS

Mitochondrial membrane potential measurements with melatonin treatment

In the present study, mitochondrial membrane potential was assessed using the most effective concentration at which melatonin treatment showed the highest cell viability. 500µM melatonin significantly lowered mitochondrial membrane potential in young cybrids, $p \leq 0.05$ in the galactose media. Importantly, the mitochondrial membrane potential was elevated in young cybrids in galactose medium without melatonin treatment. Conversely, melatonin treatment raised the mitochondrial membrane potential in middle-aged cybrids, $p \leq 0.05$, and in the old cybrids, $p \leq 0.05$ as shown in Fig. 1a, b and c. Thereby, exogenous melatonin can modulate the mitochondrial membrane potential in an upward or downward manner to effectively preserve cell viability in young, middle-aged and old cybrids.

Reactive Oxygen Species measurements with melatonin treatment

Previous studies have linked the accumulation of reactive oxygen species as a marker for tissue degeneration and dysfunction. In this study, increased levels of reactive oxygen species were observed in the young cybrids ($p<0.05$) with melatonin treatment (Figure 2a). Interestingly, the young cybrids also showed significantly higher cell viability with melatonin treatment. This finding supports a recent shift in what was earlier believed to be linked to cell dysfunction: that the presence of high levels of reactive oxygen species does not correlate with cell dysfunction during aging. Conversely, melatonin treatment in middle-aged and old cybrids lowered the levels of reactive oxygen species, $p \leq 0.05$ (Fig. 2b and 2c).

ATP levels in young, middle-aged and old cybrids with melatonin treatment

In the young cybrids, exogenous melatonin (500µM) preserved ATP levels, which did not differ significantly from untreated cybrids in galactose media only, $p>0.05$ (Figure 3a). Conversely, melatonin treatment significantly raised ATP levels in middle-aged and old cybrids, $p<0.05$ (Figure 3b and 3c). Although melatonin treatment in young cybrids produced high levels of reactive oxygen species, yet the young cybrids were able to upregulate the glycolytic pathway to increase the production of ATP through oxidative phosphorylation. Furthermore, as shown in Figure 1a, exogenous melatonin lowered mitochondrial membrane potential without compromising ATP production in the young cybrids, thereby making the entire energy making process more efficient.

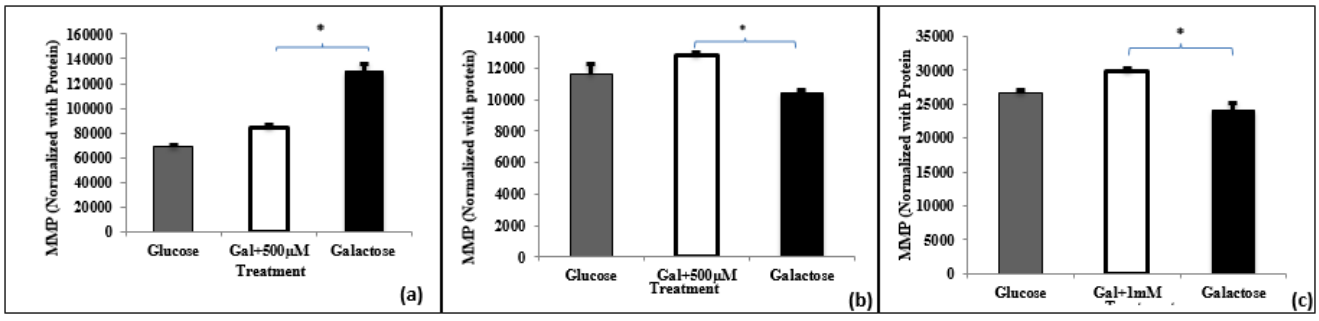

Fig 1(a): Mitochondrial Membrane Potential (MMP) normalized with total protein estimation in young cybrids, S-Y-24, in glucose media, galactose media + 500µM melatonin, and galactose media (Sensitivity 75). Young cybrids in galactose media with melatonin treatment showed a reduction in mitochondrial membrane potential, n=6, *p=3.0x10^{-5} (*p≤0.05); (b) MMP normalized with protein estimation in middle-aged cybrids, S-M-29 in Glucose Media, Galactose media + 500µM melatonin, and galactose media (Sensitivity 75). Middle-aged cybrids in galactose medium with melatonin treatment showed a significant increase in mitochondrial membrane potential, n=6, *p=1.20 x 10^{-6} (*p≤0.05); (c) MMP normalized with protein estimation in old cybrids, S-O-48 (Sensitivity 75). Old cybrids in galactose media with 1mM melatonin treatment showed a significant increase in mitochondrial membrane potential, n=4, *p=0.000145 (*p≤0.05).

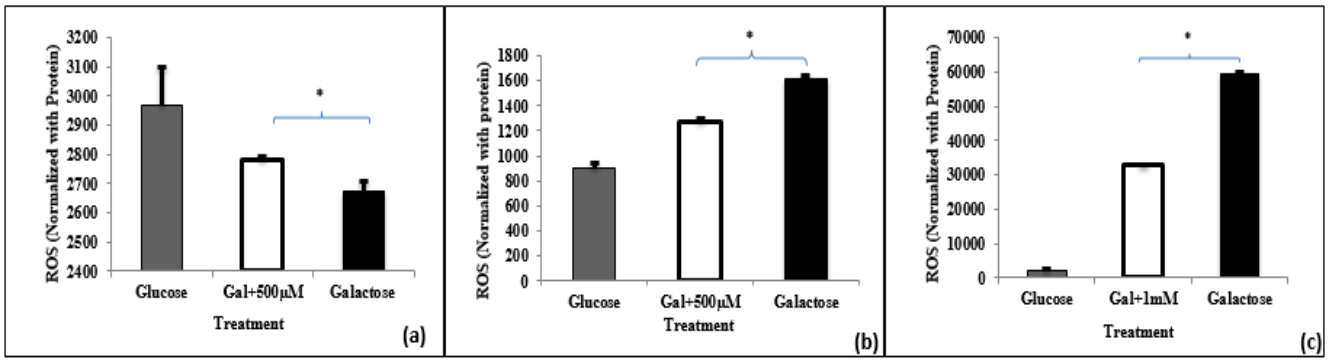

Fig 2: (a) Reactive Oxygen Species (ROS) levels normalized with protein estimation in young cybrids, S-Y-24 (Sensitivity 75). ROS levels in young cybrids in galactose media with melatonin treatment showed a significant increase in reactive oxygen species, n=6; p=0.00519 (*p<0.05); (b) ROS normalized with protein estimation in S-M-29 (Sensitivity 75). Middle-aged cybrids in galactose media with melatonin treatment showed a significant reduction in reactive oxygen species level, n=6, p=6.43 x10^{-6} (*p<0.05); (c) ROS levels normalized with protein estimation in old cybrids, S-O-48, (Sensitivity 75). Old cybrids in galactose media with melatonin treatment showed a significant reduction in ROS level compared with cybrids in galactose media only, n=4, *p=4.49 x10^{-8} (*p<0.05).

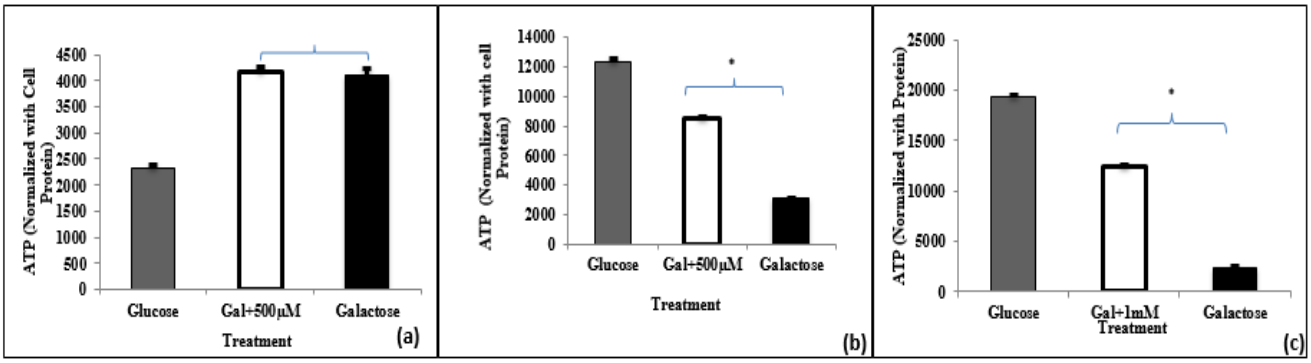

Fig 3: (a) ATP levels normalized with protein estimation in young cybrids, S-Y-24, (Sensitivity 200). Young cybrids in galactose medium with melatonin treatment showed no significant change in ATP level, n=6, p=0.3384 (p>0.05). (b) ATP normalized with protein estimation in middle-aged cybrids, S-M-29, (Sensitivity 200). Middle-aged cybrids in galactose medium with melatonin treatment showed significant increase in ATP level, n=6, *p=1.762 x 10^{-8} (*p<0.05). (c) ATP levels normalized with protein estimation in old cybrids, S-O-48, (Sensitivity 200). ATP level in old cybrids in galactose medium with melatonin treatment showed significant increase, n=6, *p=6.6x 10^{-8} (*p<0.05)

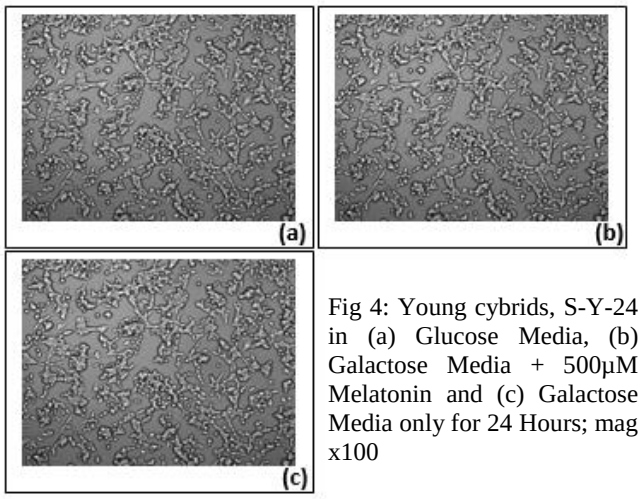

Fig 4: Young cybrids, S-Y-24 in (a) Glucose Media, (b) Galactose Media + 500µM Melatonin and (c) Galactose Media only for 24 Hours; mag x100

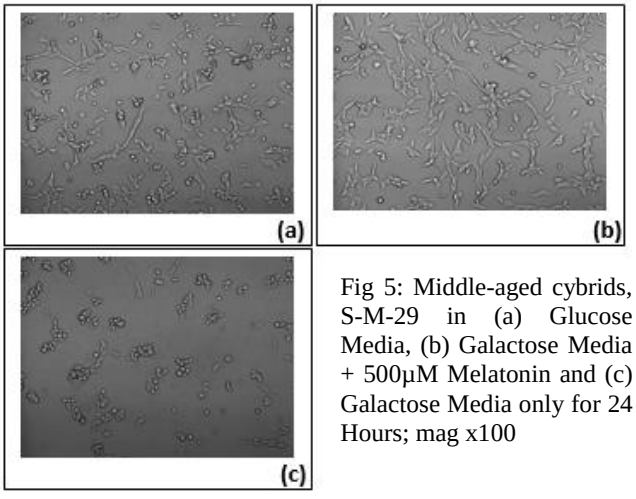

Fig 5: Middle-aged cybrids, S-M-29 in (a) Glucose Media, (b) Galactose Media + 500µM Melatonin and (c) Galactose Media only for 24 Hours; mag x100

Results of Light Microscopy Studies

Light microscopic investigations on the cells (cybrids) showed distinct characteristic pattern of cell protection occurred with melatonin treatment during normal ageing. In the young cybrids, evidence of cell death was not noticeable in the galactose medium with or without melatonin treatment; the young cybrids were preserved and were well attached after 24 hours of incubation in media (Figure 4 a, b and c).

In the middle-aged cybrids, 500µM, melatonin preserved cybrids from cell death compared with cybrids in galactose media only, Figure 5 a, b and c. Cybrids treated with melatonin are seen attached to the culture plates just as the cybrids in the control media –

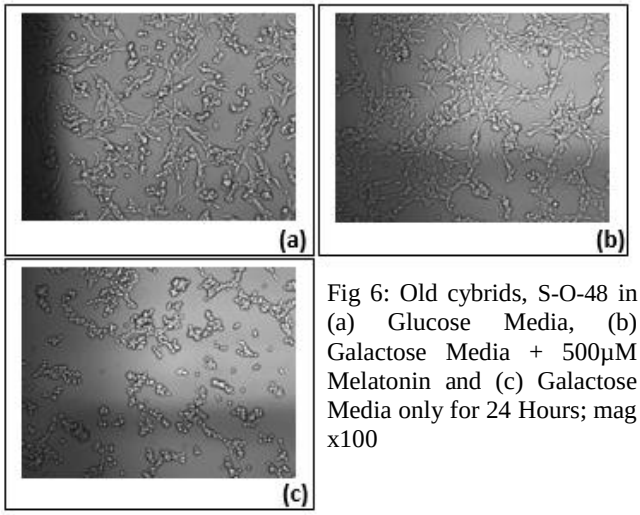

Fig 6: Old cybrids, S-O-48 in (a) Glucose Media, (b) Galactose Media + 500µM Melatonin and (c) Galactose Media only for 24 Hours; mag x100

glucose media. The round cells seen under the microscope in the galactose media represent dead cells.

The old cybrids also show cell protection with 1mM melatonin treatment in galactose media, Figure 6 b and c. Cybrids (cells) are seen attached to the culture plates in the melatonin treated cybrids. Compared with the cybrids grown in the control media (Figure 6 a), cells in melatonin treated galactose media showed similar patterns that confirm cell protection.

DISCUSSION

The role of melatonin in mitochondrial function in cybrids gives more insight into the debate about its anti-ageing properties**.** Previous studies have reported on the protective role of melatonin in cells (Li et al., 2005; Yang et al., 2011), however a growing area of interest is the mechanism by which melatonin performs its anti-ageing action. Based on the results of this study, there are further evidences of a strong relationship between melatonin administration, mitochondrial function and aging.

In the MTT assay, it was observed an increased concentration of melatonin was required to improve mitochondria function and raise cell viability from 500µM to 1mM in the cybrids in the old cybrids. This implies that the old cybrids required more melatonin to prevent cell death. It is speculated that there may be the involvement of mitochondrial melatonin receptors, which may have been reduced, in the cell response to melatonin treatment. Given that the young and middle-aged synaptosomes required lower doses melatonin to raise mitochondria function, the presence of functional melatonin receptors on their mitochondria may be a contributing factor for this finding. In contrast, the old synaptosomes required a higher concentration of melatonin (1mM) to raise mitochondria function. This finding is supported by the localization of melatonin receptors on the mitochondria membrane in brain cells of mice (Wang et al., 2011) and another observation that cells from old animals have been shown to be less responsive to melatonin treatment (Dragicevic et al., 2011).

The galactose medium is an appropriate selective medium to test mitochondrial function in cells or cybrids because mitochondria defective cells cannot produce ATP via oxidative phosphorylation in galactose medium, which will result in cell death. Hence, cells or cybrids with known mitochondria dysfunction cannot survive in the galactose medium beyond forty-eight hours. However, cells with functional mitochondria can produce ATP in the galactose medium and survive beyond 48-hours (Guy et al., 2002; Bai et al., 2005). In this study, the young synaptosomal cybrids (with functional mitochondria) were able to survive in the galactose medium and optimize ATP synthesis, while middle-aged and old cybrids were not able sustain ATP synthesis to survive in the medium beyond forty-eight hours.

The mitochondrial theory of ageing, which is widely reported as the basis for ageing proposed, that the by-products of oxidative phosphorylation - the free radicals are responsible for cell damage, mtDNA mutation and loss of cellular function (Harman, 1965). However, emerging evidences and reports have shown that the mtDNA mutation is the primary cause of respiratory chain dysfunction and not elevated changes in ROS levels (Kujoth et al., 2005; Bratic and Larsson, 2013).

The ROS levels may not be an indication of cell death or oxidative stress as ROS levels are also high in active cells with optimum mitochondrial function. We observed high ROS levels in cells with functional mitochondria (owing to their high ATP levels) in the young cybrids S-Y-24 treated with 500µM melatonin. A significant high level ROS in the case of the young cybrids treated with melatonin directly varied with large number of attached cells with functional mitochondria and an efficient production of ATP. Interestingly, the ATP generated in the melatonin treated young cybrids did not significantly differ from the untreated cybrids in galactose medium.

Mitochondrial Membrane Potential is the potential generated as electron move across the respiratory chain. The collapse of the MMP precedes cell death. Melatonin has been reported to have a dual regulatory role on the MMP such that the MMP can be raised or reduced beyond a threshold level to optimize function (Tan *et al*., 2013). An interesting finding in this study was that 500µM melatonin significantly lowered MMP in the young cybrids S-Y-24 in galactose media without compromising ATP production. In this way, melatonin at 500 µM made the entire energy-making process efficient in the young cybrids. Conversely, in the middle-aged and old cybrids, melatonin treatment raised the MMP and raised the ATP levels.

Light microscopic investigations on the cells showed distinct characteristic pattern of cell protection occurred with melatonin treatment during ageing. This

suggests that the young cells were able to upregulate the glycolytic pathway to produce ATP through oxidative phosphorylation which was essential for cell survival. This finding was supported by the finding that the younger cybrids were still attached in galactose medium after 24 hours. In the middle-aged cybrids, the cells treated with 500µM melatonin show noticeable structural integrity than the cells in galactose medium. The cells in galactose medium had undergone observable cell death within 24 hours after treatment. This implies that the cells were not able to up-regulate the glycolytic pathway to use oxidative phosphorylation to make ATP as ageing sets in. Similarly, there was significant cell death in the old cybrids in the galactose medium within 24 hours. Considerable cell death was observed in the old cybrids which were cultured in galactose medium for 24 hours but melatonin treatment preserved the old cybrids from cell death.

The fluorescence microscopy study with Hoechst and TMRM stains showed robust nuclear (blue stain) and mitochondrial (red stain) activity respectively in the old cybrids treated with melatonin compared with the untreated cells. Therefore, melatonin preserved the old cybrids nucleus and mitochondria.

From the results of this study, it is concluded that melatonin is capable of altering mitochondria bioenergetics to raise ATP production during ageing. It can also be inferred that exogenous melatonin administration before the onset of age-related decline in mitochondria function may be may be beneficial to cells by increasing cell response to melatonin. Melatonin may be a beneficial therapeutic agent during normal ageing, preventing events leading to neuronal mitochondria dysfunction.

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