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Research Article

Chronic administration of Melatonin protects D-galactose-induced impairment of cholinergic system in brain of *Musca domestica* (L.)

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Abstract

D-galactose (D-gal) administration is responsible for augmented ROS production and consequences in impairment of cholinergic system. Melatonin is well known for itspotent antioxidant and free radical scavenging activity. In this study, we evaluated the protective effect of Melatonin upon chronic administration (20 days) against D-gal-induced impairment of cholinergic system in male housefly, *Musca domestica*. Our results demonstrated that Melatonin administration significantly improved locomotarybehaviorof D-gal-treated houseflies in climbing activity test. One of the potential mechanismsof this action was decreased AGEs, ROS and protein carbonyl levels in the brain of D-gal-treated male houseflies. Furthermore, our results also showed that Melatonin significantly inhibited cholinesterase (AchE) activityin brain of D-gal-treated male houseflies, which could help restore impairment of brain function.

INTRODUCTION

According to Troen (2003), aging is a slow and gradual biological process, associated with various morphological and biochemical changes in biological system. Brain senescence plays an important role in cognitive dysfunction frequently foundlinked with various neurodegenerative disorders. Crivello *et al*., (2005) proved that many age-related behavioral changes in motor and cognitive performance happenstill in the lack of definite, age-related, neurodegenerative diseases like Alzheimer disease or Parkinson disease. Melatonin is well known for its potent antioxidant and free radical scavenging activity. D-galactose (D-Gal) is a reducing sugar which in normal concentration can be metabolized easily. Conversely, when given at high levels, it forms advanced glycation end products (AGEs) by reacting with the free amines and amino acids of proteins and peptidesin vivo to (Lu *et al*., 2007; Tian *et al*., 2005). There are considerable evidences which reveal that AGEs which in turn causes activation of receptor for advanced glycation end products (RAGE) and can induce ROS production and also increase protein carbonyl levels, which can elicit aging process by triggering the early phases of age-related diseases (Cai *et al*, 2006; Srikanth *et al*., 2011; Tian *et al*., 2005, Kumar *et al*., 2011). Additionally, current findings have confirmed chronic supplementation of D-Gal in rodents leads to the decreased expression of memory-related protein. It also causesweakening of learning and memory function foundallied with mutilation of the basal forebrain cholinergic system (Cui *et al*., 2006; Lei *et al*., 2008; Lu *et al*., 2006; Lu *et al*., 2009).Consequently, much of the research on cognitive decline has focused on the cholinergic system (Bacciottini *et al* 2001), and the treatment for refurbishing the cholinergic neurotransmission. D-Galactose (D-Gal) -induced aging models in

Drosophila, houseflies, mice and rats have been widely used; however, the underlying mechanisms are poorly understood. In Indian context, very little work has been done on D-gal induced damage in insects (Gaikwad *et al*., 2010). Additionally, more emphasis is given only on the oxidative damage caused by D-gal. To date, little is known about the influence of D-Gal -induced aging on neural damage in insects.

Melatonin, the main secretory product of the pineal gland, is a direct free radical scavenger and a natural antioxidant, which protects against oxidative damage caused by variety of toxicants. Dietary intake of melatonin had shown to extend life span as well as its resistance against paraquat induced oxidative stress in *Drosophila*. (Binilla *et al*, 2001). The therapeutic roles of melatonin in insomnia, mood disorders and AD have been extensively studied (Srinivasan et al. 2012). But itsprotective effect chronic administration against D-Gal-induced impairment of cholinergic system in insects is not yet studied.

Therefore, the present study has been designed toexplore the possible role of melatonin against D-galactose-induced locomotary behavior, oxidative damage and cholinesterase (AchE) activity in brain of male houseflies. Till now, no work has been done to study whether melatonin has an effect against D-gal-induced impairment of cholinergic system in housefly model. In the present study, we addressed this issue and investigated the potential mechanism underlying its action.

MATERIALS AND METHODS

Fly stock rearingand administration:

Rearing of *Musca domestica*:

Musca domestica is a holometabolous insect; its lifespan includes egg, larval instar, pupal and adult stages. Under normal culture conditions 26 ±1°C and 70% humidity, the average adult (post-eclosion) lifespan of wild type flies is between 60-70 days.

Musca domestica administration:

M. domestica stock cultures were maintained on the standard *M. domestic* medium as described by Szczerbina, et al., 2008 with minor modifications. Newly eclosed flies were immobilized on ice, divided into treatment groups, and allowed to regain consciousness. All treatment groups were fed on 1 gm dry milk powder in a clean and sterile 35 mm Petri dish. In another Petri dish cotton soaked in 2% sucrose solution prepared in distilled water was placed in the jar as a source of water for flies. All the treatments are added in the drinking water. Based on earlier standardized experiments (data not shown), maleswere randomly selected into four groups (n=20) as follows.

1. Control Group: a Normal control group; houseflies were fed on 2% D-sucrose

2. D-Gal Group: a D-galactose model control group; houseflies were fed by 2% D-galactose

3. D-Gal + MEL Group: Melatonin group; houseflies were fed by 2% D-sucrose + 1mM melatonin

4. MEL Group: a positive Melatonin group where in addition to receiving D-galactose, the houseflies were also supplemented with 1mM melatonin

Behavioral tests:

This is the first report of climbing activity assay in *M. domestica.* This method was modified based on the earlier reports on *Drosophila melanogaster* (Le Bourg and Lints, 1992; Jordens et al, 1999 and Bauer *et al*, 2004) which is a popular model for this kind of study.Based on previous findings that aging promotes decrease in physical activity in *M. domestica* (Ragland and Sohal ,1973; Agarwal and Sohal, 1994; Yan and Sohal, 2000) in this study climbing activity (CA) has been examined as a potential marker of advanced aging. To account for the daily mortality rate of *M. domestica*, each climbing activity assay value was expressed as a percentage of total remaining flies in their respective jars (% CA). These % CA values (10 values/ experimental day/ trial) were averaged into one % CA value / trail day. The average % CA was determined for each treatment group.

Climbing assay was performed according to (Feany and Bender 2000) with some modifications. In brief, 10 male houseflies were placed in a tube, and theflies were given 10 s to climb up the tube. At the end of each trial, the number of housefliesthat climbed up a vertical distance of 20 cm ormore was recorded. Each trial was triplicated. Flies were testedevery 3 days and the number of survived houseflieswas also recorded.

Preparation of tissue Sample:

After 20days of treatment, the houseflies were immobilized on ice for 10 minutes and sacrificed for the further experiments. The brains were quickly dissected and homogenized with ice-cold 50 mM phosphate-buffered saline (pH 7.4). The resultant homogenate was centrifuged at 10000 rpm for 10 minutes and stored at -80 °C for biochemical analysis. On the day of assay, a 10% (w/v) tissue homogenate was prepared in 50 mM phosphate-buffered saline (pH 7.4) using glass homogenizer.

The homogenate was centrifuged at 4000 rpm for 10 min at 4°C and the supernatant was used for further analysis.

Biochemical analysis:

AGE-enzyme-linked immunosorbent assay:

Quantitative measurement of AGEs was performed by AGEs and TNFR1 were measured in brain homogenates by ELISA (Cell Biolabs, Inc. San Diego, CA, and USA) kit as per the instructions given absorbance (OD) was taken using 450 nm as the primary wave length on a microplate reader. AGE-BSA Competitive ELISA Standard Curve was generated as an AGE standard curve. Sample AGE values were calculated from the standard curve and expressed as µg/ml.

Assay of ROS:

ROS was measured using dihydrodichloro fluorescein diacetate (H2 DCFH-DA), a non-polar compound, which after conversion to a polar derivative by intracellular esterases, rapidly reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF) (Chandrashekar and Muralidhara, 2008).The ROS generation was calculated from a DCF standard curve and expressed as qmol DCF/min/mg protein.

Protein carbonyl assay as Oxidative stress marker:

The protein carbonyl (PCO) levels in brain and flight muscles samples of all groups were spectrophotometrically measured as per the Reznick and Packer method (1994). In this assay the Protein carbonyls react with 2, 4-dinitrophenylhydrazine (DNPH) and generate chromophoricdinitrophenylhydrazones. The resultant absorbance was measured at 360 nm and PCO levels were calculated as nmol/mg protein by using the molar extinction coefficient of DNPH, ε = 22,000*M*/cm.

Estimation of Acetyl cholinesterase (AChE) activity:

AchE is a marker of loss of cholinergic neurons in the forebrain. The AchE activity was assessed by Ellman *et al* (1961) method. AChE activity was calculated by using molar extinction coefficient of 5- thio-2-nitrobenzene acid at 412 nm. These results were expressed nmoles of substrate hydrolyzed/min/mg protein.

Total Protein Assay:

Protein concentrations in all test samples were determined by using Bradford reagent (Bradford, 1976). The amount of protein was quantified using bovine serum albumin as the standard.

Statistic analysis

All statistical analyses were performed using the Graphpad Prismsoftware, version5.00. Group differences in all data were analyzed with one-way ANOVA followed by Tukey's HSD post hoc test. Data were expressedas means ± SEM. Statistical significance was set at $p < 0.05$.

RESULTS DISCUSSION

1. Melatonin counteracts locomotory impairment of D-gal-treated houseflies:

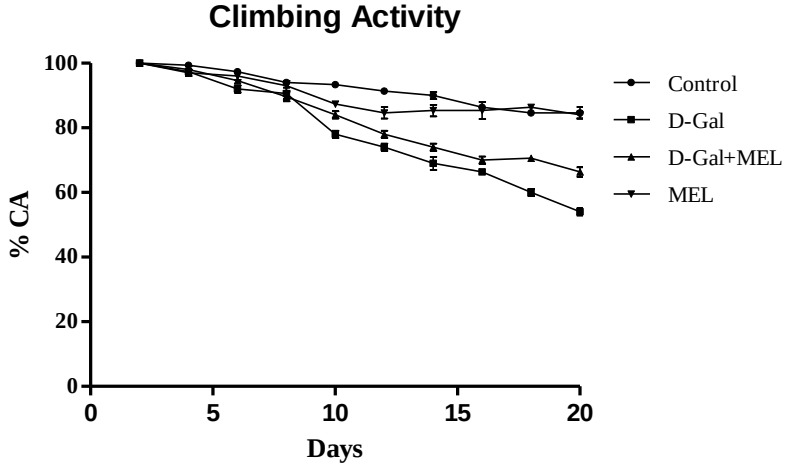

Fig.1. Melatonin suppresses locomotory dysfunction in the form of climbing activity in the D-gal-treated male houseflies:

Results are mean ± SE (Standard Error) of three different set of observations. (n = 30).

Co-treatment of MEL (D-Gal+MEL group) rescued the D-Gal induced locomotory dysfunction. ($p < 0.001$; ANOVA; $n > 100$).

Locomotor assay is a behavioral paradigm to assess theneural functional abnormalities based on the negative geotaxis against gravity. D-Gal treated group showed decrease in % climbing activity. Houseflies co-treated with D-Gal showed an early decline of climbing ability (starting from 10 days) when compared with control flies. We found MEL co-treatment improved the climbing ability ofthese D-Gal treated houseflies significantly after 20 days compared to the control group (Fig. 1, $P < 0.001$). However, no obvious difference was observedbetween the treated and non-treated groups at timepoints of 2to8 days (Fig. 1).

2. Melatonin reduces AGEs, ROS and protein carbonyl levels in the brain of D-gal-treated houseflies: As seen in Fig. 2, D-Gal administration significantly increased AGEs, ROS ($p < 0.01$); and protein carbonyl levels in the brain ($p < 0.001$), as compared to the control group. The data indicated that oxidative stress *in vivo* was elevated in the brain of D-Gal-treated houseflies. Remarkably, Melatonin could decrease AGEs, ROS and protein carbonyl levels in the brain ($p < 0.01$). There was no significant difference among the control group, the MEL group and the D-Gal+MELgroup in AGE and ROS except Protein carbonyl level ($p < 0.05$).

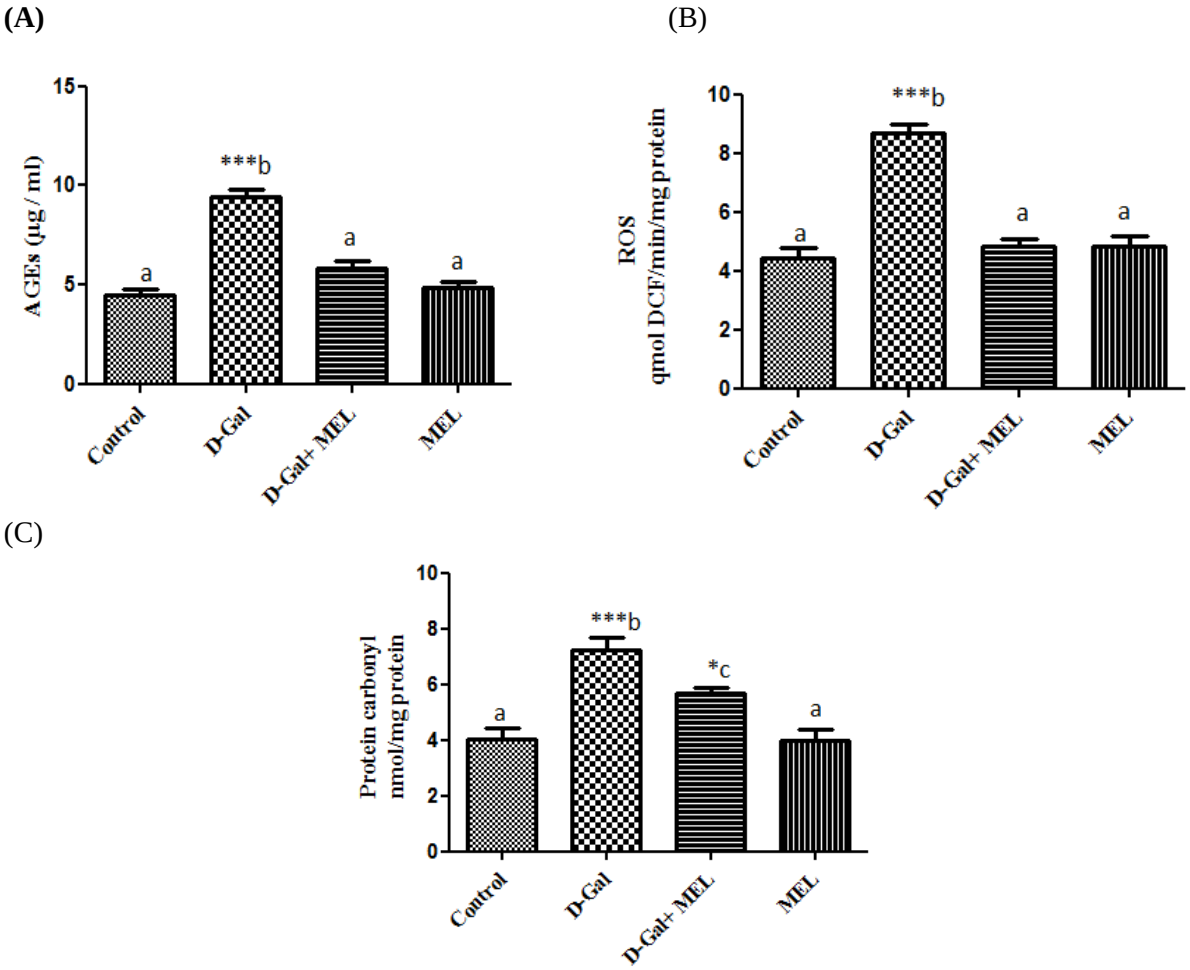

Fig. 2. Melatonin reduces AGEs, ROS and protein carbonyl levels in the brain of D-gal-treated houseflies:
Results are mean ± SE (Standard Error) of three different set of observations. (n = 20).
(A) Comparison of AGEs levels (µg/ml) in brain.
(B) Comparison of ROS levels (as qmol DCF/min/mg protein) in brain
(C) Comparison of protein carbonyl levels (nmol/mg protein) in brain
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control group;
Different alphabets (a, b and c) indicate significant difference between respective group ($P < 0.05$)

3.

Melatonin reduces AchE activity in the brain of D-gal-treated houseflies:

Compared to control group, D -Gal group displayed a remarkable increase of AChE activity ($P < 0.001$) (Fig. 3) which indicated cholinergic system in D-gal-treated houseflies was impaired. Compared to D -Gal group, D-Gal+MEL group showed decreased AChE activity ($P < 0.05$) However, there was no significant difference of two indexes between Control and MEL groups.

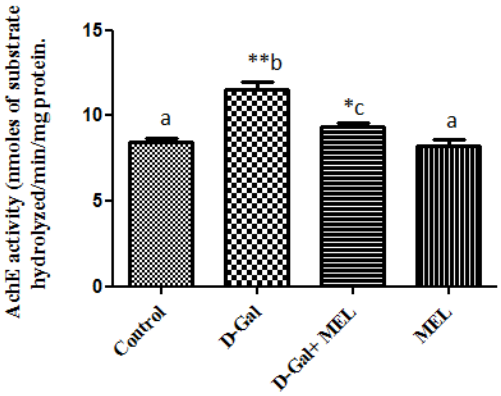

Fig.3. Melatonin decreases AchE activity in the brain of D-gal-treated houseflies:
Results are mean ± SE (Standard Error) of three different set of observations. (n = 20).
AchE values are expressed as nmoles of substrate hydrolyzed/min/mg protein
*$P < 0.05$, **$P < 0.01$ vs. control group;
Different alphabets (a, b and c) indicate significant difference between respective group ($P < 0.05$)

D-Gal is a reducing sugar proved to react freely with the free aminesof amino acids in proteins and peptides both in vivo and in vitro toform AGEs. Noticeable findings also proved that AGEs is a harmless post-translationalprotein modification and it can accelerate the aging process (Srikanth *et al*., 2011). One of the impending mechanisms of AGE-induceddamage is reactive oxygen species (ROS) production, especiallysuperoxide and hydrogen peroxide release stimulated by AGEs (Srikanth *et al*., 2011). Studies reported by Fan et al., 2009; Lu et al., 2006, 2007; Wu *et al*., 2008 have confirmed that long-term injection of D-gal in mouse brain induces overproductionof ROS directing neuronal oxidative damage. Simonian and Coyle, 1996 and Lei *et al*., 2008 also supported this by recommending the fact that oxidative damage is an crucial source of aging related neurodegenerative diseases such as Parkinsons disease (PD) and AD.in this study, we also found similar results by increasing AGEs by D-Gal supplementation in houseflies. We also found that melatonin significantly reduces ($p < 0.05$) this profound effect (Fig.2, A).

Along with inducing ROS (Fig.2, B), D-Gal formed AGEs can also add protein carbonyl production (Cai *et al*., 2006; Lu *et al*., 2007; Tian *et al*., 2005) we found similar results as seen in (Fig.2, C). We also found this increased ROS level in D-Gal treated group significantly ($p < 0.05$) whereas significantly reversed ($p < 0.05$) this effect (Fig.2, B).

Muthuraju *et al*., (2009) reported that learning and memory deficits in a various neurodegenerativedisorders are correlated with degeneration of cholinergic neurons. In the present study, cholinergic markers like AchE levels in the houseflybrain are studied (Fig. 3). Results demonstratedthat D-Gal administration persuaded a significant increase in AchE activity which would damage locomotory behavior in houseflies as seen in Fig. 3. Conversely, Melatonin could significantly reverse the increased AchE activity co-treated with D-Gal (D-Gal + MEL group) thereby increasing the climbing activity significantly (Fig. 1).

CONCLUSION

Melatonin could be involved in stabilizing synaptic connections. Therefore, it has the potential to reduce brain damage and improve locomotory deficits in mimetically aged male houseflies related to changes in the cholinergic system. In addition, melatonin also has anti-oxidative properties as it is a potent antioxidant. The potential mechanisms causal the neuroprotective effect of melatonin in the D-gal-treated houseflies might be decreasing AGEs, ROS and protein carbonyl levels.

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