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Antioxidant Effects of Melatonin and R-α-lipoic acid on Mimetically Aged Houseflies

A. A. Sharbidre
Department of Zoology, Savitribai Phule Pune University, Pune, Maharashtra, India

ABSTRACT

D-galactose (D-gal) has been widely used to induce mimetic aging in various animals. In the current study, we used Melatonin and R-α-lipoic acid to explore their antioxidant and anti-aging effect in D-gal induced mimetic aging in male Musca domestica. In this study, we finalized the doses of Melatonin and R-α-lipoic acid upon chronic administration (20 days). Our results confirmed that these antioxidant administrations significantly improved the ROS generated by decreasing the levels of AOPPs, and MDA levels in aging houseflies. Melatonin and R-α-lipoic acid administration elevated the activities of SOD and proteins levels which are suppressed by aging. Our results signify that both Melatonin and R-α-lipoic acid effectively alleviated oxidative stress and improved protein levels in D-Gal induced aged houseflies.

Keywords: Aging; D-galactose; oxidative stress; Musca domestica; Melatonin; R-α-lipoic acid

I. INTRODUCTION

Aging is the progressive accretion of alteration over time which is allied with or accountable for the increasing susceptibility to disease and death. D-Galactose (hereafter referred as D-Gal) accelerated aging models are found have tremendous similarity with normal aging models thus and extensively used for study of aging, for assessing various agents used for slowing down the effects of aging and related diseases.

Musca domestica L. 1758 is considered as excellent model system to explore longevity promoting qualities of neutraceutical extracts and phyto-constituents (Toroser and Sohal, 2005). This aging model might be valuable in spotting the role of efficient antioxidants and underlying mechanisms in retarding aging (Cui et al., 2004).

Melatonin (N-acetyl-5-methoxy-tryptamine, hereafter referred as MEL) is a main product secreted by the pineal gland using tryptophan. MEL has been reported to minimize the accretion of lipid per oxidation generated products. As it is not showing any pro-oxidant and lethal effects, this hormone is proved to possess a great radical scavenging property.

R-α-lipoic acid (hereafter referred as LA) is a naturally occurring component, essential for the function of different enzymes that take part in mitochondria's oxidative metabolism. Because of this property, LA is commonalty used in multivitamin preparations, anti-aging drug supplements, and also added in pet food (Shay et. al, 2009). LA is a potent biological antioxidant with a scavenging capacity for many reactive oxygen species (Scott, et. al, 1994; Packer et.al, 1995).

As till date there is no report on *M. domestica* oxidative stress during mimetic aging, the current study was conceded out to check the hypothesis that the oxidative stress elicited by the ingestion of D-Gal is associated with antioxidant defense.. The effect of MEL and LA acid supplementation on *M. domestica* aging is not so far studied. Therefore this is the first effort to study such effect. In current investigation the male houseflies were used to study whether D-Gal- induces oxidative stress and can it be minimized by the antioxidant properties of MEL and LA.

II. Materials and Methods

Based on our previous standardization experiments of MEL and LA supplementation; 1mM MEL and 0.5 mM LA were found to be most suitable to minimize the adverse effects of D-Gal on *M. domestica* life span i.e. these two doses significantly increased the life span of *M. domestica* when co-treated with D-Gal (unpublished

data). To ensure further and to confirm that whether these concentrations are actually minimizing the stress, we made following six experimental groups of houseflies which were randomly selected into six groups (n=4).

Group I: a Control group (C); houseflies were fed on 2% D-sucrose
Group II: a D-Gal model control group; houseflies were fed by 2% D-galactose
Group III: MEL group; houseflies were fed by 2% D-sucrose + 1mM melatonin
Group IV: LA group; houseflies were fed by 2% D-sucrose + 0.5 mM LA
Group V: a positive MEL group (D-Gal + MEL) where in addition to receiving D-Gal, the houseflies were supplemented with 1mM MEL
Group VI: a positive LA group (D-Gal + LA); in addition to receiving D- Gal, the houseflies were fed a diet supplemented with 0.5 mM LA

We used the technique reported by Aksu et al, (2014) was used with some modifications for standardization and dose selection experiment. Both male and female individuals (n=2, each) were kept in single experimental jar for 5 weeks under normal culture conditions i.e. 26 ± 1°C and 70% humidity. At the end of this period the experiment was concluded. During this phase, the houseflies enter in middle age. Only males were isolated and and used for further biochemical estimations. We discarded female houseflies as there might be probable role of estrogens as antioxidants (Altun et al., 2011). Each experiment was repeated thrice.

Gustatory assay:
Assessment of food intake by house fly was done by adding visible dye into diets, and after that gut redness was measured (Cui et al., 1998). Sulforhodamine B was used to detect the amount of food consumed by flies. Houseflies were starved for 24 hours on tissue paper soaked with distilled water. After starvation period, they were again transferred back on their respective experimental set. Food i.e. the drinking water was mixed with 2% sulforhodamine B. They were allowed to feed for 3 hours on the dye-mixed food. After 3 hours of feeding, flies were immobilized by placing on ice, and dissected. Their entire gut region of was dissected and homogenates were prepared using the 0.1 M phosphate buffer, pH 7.2. Absorbance of supernatant was measured at 540nm.

Biochemical Methods:
After 20 days of treatment male houseflies (n=10 per group) were exposed to -80 °C for 15 minutes and their heads were dissected. We used ice-cold 50 mM sodium phosphate buffer (pH 7.0) for homogenization of heads facilitated by sterilized motor driven tissue grinder (Genetix Biotech). Subsequently, the homogenates were subjected to centrifugation at 10,000 rpm for 10 minutes (0 °C) and the resultant supernatants were kept at -80 °C. These supernatants were used for further biochemical estimations.

Quantification of Advanced Oxidative Protein Products (AOPPs):
For this assay, the method reported by Hanasand et al., (2012) with some modifications was used for spectro-photometric analysis of AOPPs. The reaction mixture absorbance was instantly read at 340 nm. Concentrations of AOPP were determined using Chloramine Standard curve and calculated as µM / lit of chloramines-T equivalents.

Estimation of Thiobarbituric Acid Reacting Substances (TBARS):
The levels of lipid peroxidation was estimated by measuring the TBARS content as per the procedure of Ohkawa et al., (1979) with minor modifications.

Superoxide Dismutase Activity (Cu,Zn-SOD):**dismutase activity** (EC – Number 1.15.1.1) **assay**:
Cu,Zn-SOD (EC1.15.1.1) activity was measured by inhibition of formazon formation in the presence of enzyme (Beauchamp and Fridovich, 1971). The enzyme activity was calculated in terms of Units/mg of protein.

Quantification of total Protein:
Protein concentrations in all test samples were determined by using Bradford reagent (Bradford, 1976) with some modifications with bovine serum albumin (BSA) as a standard.

III. Results

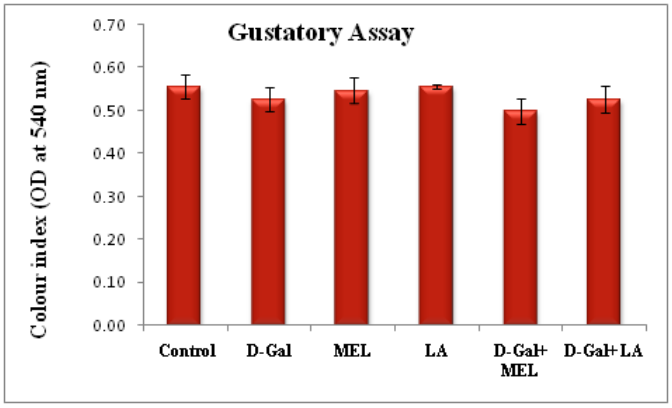

Figure 1. Gustatory (feeding) assay

Above figure shows that the all the six groups of houseflies did not show significant difference in their feeding assay (Figure 1). Thus the results obtained from the following experiments are not due to the alteration in the feeding behaviour of the houseflies, but it was due to the presence of the treatment compound itself.

Table 1. Effect of MEL and LA on D-gal-induced aging related parameters in male houseflies.

Treatment	AOPPs levels µM / lit of chloramines-T equivalents (% of control)	LPO nmoles MDA per gm of tissue (% of control)	Cu,Zn-SOD Units / mg of protein (% of control)	Total Protein mg / ml BSA (% of control)
Control	99.18 ± 13.86 (100)	0.178 ±0.05(100)	46.362±3.16(100)	0.109 ± 0.04 (100)
D-Gal	212.6 ±52.76 [a](214.36)	0.585 ±0.06a (328.65)	15.628±2.15 [a](33.71)	0.087 ± 0.018 [a](79.82)
MEL	97.44 ± 23.96 (98.92)	0.173 ± 0.03 (97.19)	45.86±3.12 (98.92)	0.103 ± 0.03 (94.50)
LA	108.2 ± 17.15 [b](109.09)	0.169 ± 0.04 [b](94.94)	46.212 ± 4.11 (99.68)	0.105 ± 0.023 (96.33)
D-Gal +MEL	144.2 ± 22.15 [c](145.39)	0.453± 0.06 [b,c] (254.49)	21.93 ±2.049 [c](47.30)	0.0927 ± 0.018 [a](85.05)
D-Gal +LA	120.9± 23.77 [c](121.89)	0.305 ±0.06 [c](171.35)	25.872 ±2.05 [b,c](55.80)	0.0979 ± 0.018 [a](89.82)

Values are mean ±S.E.M. [a]P<0.05 as compared to Control group;
[b]P<0.05 as compared to D-gal-treated group;
[c]P< .05 as compared to D-Gal +MEL and D-Gal +LA to D-gal group
(repeated measures one-way ANOVA followed by Tukey's test for multiple comparisons).

As shown in Table 1, compared to Control group houseflies, chronic administration of D-Gal significantly increased AOPP and MDA concentration, whereas resulted in depletion of superoxide dismutase, and total protein level (P <0.05).

As compared to control group, D -Gal group displayed a remarkable increase of AGEs levels (p< 0.001) (Table 4.4.) indicating elevation of *in vivo* oxidative stress in the brain of D-Gal-treated houseflies. Remarkably, Melatonin and LA could decrease brain AGEs levels when compared with D -Gal group, similarly D-Gal+MEL as well as D-Gal+LA group showed decreased AGEs levels (p< 0.05). However, no significant difference was found in two indexes between Control and MEL groups.

However, chronic co-treatment of both MEL and LA significantly relieved the oxidative damage which is shown by abridged AOPP and MDA levels and refurbishment of superoxide dismutase, and total protein level as than D-gal- group levels. Further, MEL and LA treatment by itself did not show any significant changes in oxidative stress parameters of Control group houseflies (Table 4.4). These results ensured and confirmed that these concentrations are actually safe for the individual experiments.

IV. Discussion

Based this we first tried to establish the mimetic aging in houseflies caused by D-Gal. MEL and LA doses were added in the drinking water and their effect on various physiological assays like feeding rate of houseflies was studied. Analyses of feeding rates were done by gustatory assay between repetitive trials of D-Sucrose fed houseflies shown no significant differences. Likewise, there was no significant difference observed between all replicates of D-galactose-fed as well as antioxidants (MEL and LA) fed houseflies tested. Hence, it was specified that any potential effect of D-Gal as well as antioxidants (MEL and LA) on houseflies would not be due to un-even feeding.

Lots of publications revealed that the mimetic aging induced by the chronic administration of D-Gal is linked with accretion of oxidative stress. Lessened activity of antioxidant enzymes and increase in LPO are biomarkers of oxidative stress (Cui et al., 2006; Hsieh et al., 2011). Oxidative stress performs a crucial role in the age-linked cognitive decline in neurodegenerative disease like Alzheimer's (Kumar and Gupta, 2003) and Parkinson (Kaur et al., 2011) as neuronal membranes easily get damaged by free radicals. MDA is an

imperative biomarker of oxidative damage under conditions of oxidative stress (Elia et al., 2002).

Thus, these findings clearly demonstrated that, D-Galactose induces oxidative stress in the males of *Musca domestica*. This stress can be reduced by Melatonin and R-α-lipoic acid. Thus, Melatonin as well R-α-lipoic acid acts as an efficient free radical scavenger by protecting tissues and also tries to prevent D-galactose induced oxidative stress. OS is also elevated in D-Galactose induced aging process in comparison to controls. Sucrose control in response to anti-oxidant treatment over the duration of study partially restored the antioxidant defense and decreased the extent of oxidative damage in D-Galactose induced aged houseflies. This study also necessitates the importance of antioxidant therapy in aging houseflies to alleviate oxidative damage caused. Thus, these could be effectively used in the treatment of aging related disorders owing to their anti-aging and antioxidant potential.

V. Conclusion

The present study thus concludes that, D-Galactose induces oxidative stress in *Musca domestica.* This stress can be reduced by Melatonin and R-α-lipoic acid. Thus, Melatonin as well R-α-lipoic acid acts as an efficient free radical scavenger by protecting tissues and also tries to prevent D-galactose induced oxidative stress. However, the long-term effects of unbiased and antioxidant- supplemented revival on various organ functions necessitates further study.

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