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BOOSTING LIFESPAN AND ANTIOXIDANT DEFENSES: THE SYNERGISTIC POWER OF DIETARY RESTRICTION AND VITAMIN C IN DROSOPHILA MELANOGASTER

Healthcare

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ABSTRACT

Dietary restriction (DR) and antioxidant supplementation, particularly with Vitamin C (ascorbic acid), have been linked to mitigating aging effects. In this study, we examined the combined effects of DR and ascorbic acid on antioxidant enzymes and lifespan in Drosophila melanogaster. Flies were divided into four groups: control, 40% DR, ascorbic acid (AA) supplemented and combined 40% DR + AA supplemented group. Key factors, including lipid peroxidation levels and antioxidant enzymes activity, were measured over 56 days at a weekly interval. Results showed that, lipid peroxidation increased with age but were significantly lower in the experimental groups, especially in the 40% DR + AA group. Additionally, antioxidant enzyme activities, including superoxide dismutase and catalase, were significantly higher in the 40% DR and 40% DR + AA groups. All experimental groups demonstrated a greater maximum lifespan to the control. These findings suggest that, the synergy of DR and Vitamin C supplementation enhances health and lifespan more effectively than either approach alone.

KEYWORDS

Antioxidant enzymes, Ascorbic acid, Dietary restriction, Drosophila melanogaster, Maximum Lifespan Potential

INTRODUCTION

Advancements in medical science have significantly increased the human life expectancy. However, with this increase in lifespan comes a rise in the occurrence of age-related diseases and dysfunctions (Donato et al., 2018; Li et al., 2021; Trevisan et al., 2019). To address these challenges, it is crucial not only to extend lifespan but also to preserve or enhance physical health. Achieving this goal requires interventions that target the aging process itself. One of the primary culprits behind aging-related damage is the accumulation of free radicals, such as superoxide and hydroxyl radicals, which cause oxidative damage to cellular macromolecules and organelles (Davies et al., 1982; Dizdaroglu et al., 2002; Gebicki, 2016; John Aitken, 1995; Pajares et al., 2018). These damaged molecules and organelles are typically repaired or degraded within the lysosomes (Ahlberg et al., 1982; Bonam et al., 2019). However, as oxidative damage accumulates with age, the burden on the repair systems and lysosomes increases. When lysosomes fail to completely degrade and recycle damaged material, it accumulates as age pigments or lipofuscin granules, which act as additional sources of free radicals (Szweda et al., 2003; Whitworth, 2011). These free radicals interfere with cellular metabolism and ultimately lead to cell death.

To combat free radical-induced damage, cells rely on an antioxidant defense system that neutralizes these harmful molecules and reduces their destructive effects (Deruisseau et al., 2006). Unfortunately, this antioxidant defense system diminishes with age (Fusco et al., 2007), highlighting the need for exogenous antioxidant supplementation. Another promising strategy to mitigate age-related dysfunction is enhancing the cell's ability to efficiently recycle damaged components through autophagy, a process that can be stimulated by dietary restriction (DR) (Chung & Chung, 2019). Research has shown the potential benefits of ascorbic acid supplementation in addressing aging-related dysfunctions (Carr & Frei, 1999; Harrison, 2012; Rattanawiwatpong et al., 2020), and there is ample evidence suggesting that dietary restriction can slow the aging process (Forster, 2003; Kaeberlein et al., 2006; Ungvari et al., 2008). In this study, we investigated the synergistic effects of dietary restriction and antioxidant supplementation in Drosophila melanogaster to explore their potential in combating aging-related dysfunctions.

MATERIAL AND METHODS

In this study, Drosophila melanogaster (fruit flies) was chosen as the model organism to investigate the effects of various dietary interventions. The wild strain of D. melanogaster was procured from the Department of Zoology, Banaras Hindu University, Varanasi, India. The flies were maintained in a controlled environment at 21 ± 0.5°C and 65% relative humidity within a BOD incubator, and were cultured on a maize-based medium.

Newly emerged adult flies were carefully collected from culture bottles and transferred to glass vials (90mm x 35mm) for further experimental procedures. The flies were then categorized into four distinct groups for the study:

1. **Control Group:** Flies in this group were raised on a standard maize medium containing 8g sugar, 9g maize powder, 0.66g sodium benzoate, 0.66ml propionic acid, 3g agar, and 200ml drinking water. After the medium solidified, a granular form of dry yeast (3g) was evenly spread on the surface to enrich their diet.
2. **40% Diet Restricted Group (40% DR Group):** This group was fed on a maize medium with a 40% reduction in sugar and maize powder compared to the standard formulation used for the control group.
3. **Ascorbic Acid Supplemented Group (AA Group):** Flies in this group were raised on the conventional maize medium, which was supplemented with 100mM ascorbic acid, as per the methodology of Bahadorani et al. (Bahadorani et al., 2008).
4. **40% Diet Restricted + Ascorbic Acid Supplemented Group (40% DR + AA Group):** In this group, flies were subjected to a 40% diet restriction alongside the supplementation of 100mM ascorbic acid.

Estimation of Lipid Peroxidation (Wills, 1966): Lipid peroxidation levels were assessed at various time points: day 0, day 7, day 14, and continuing through day 56. To prepare the homogenate, wings and legs were removed, and the remaining tissue was homogenised in a reaction mixture consisting of 75mM potassium phosphate buffer (pH 7.04), 1mM ascorbic acid, and 1mM ferric chloride. The final tissue concentration in the homogenate was 2mg/ml. A 0.2ml aliquot of the homogenate was mixed with 0.8ml distilled water, 1ml of 20% trichloroacetic acid (TCA), and 2ml of 0.67% thiobarbituric acid (TBA). The mixture was then boiled for 10 minutes and allowed to cool. The optical density was measured at 532nm. Lipid peroxidation was quantified as nanomoles of malondialdehyde (MDA) per milligram of tissue.

Estimation of Superoxide Dismutase (SOD): Superoxide dismutase (SOD) activity was measured using the method described by Beauchamp and Fridovich (Beauchamp & Fridovich, 1971). This assay is based on the reduction of nitro blue tetrazolium (NBT) to a blue-colored formazan product by superoxide radicals, which are generated photochemically in the reaction system. The presence of SOD reduces the generation of these radicals, thereby decreasing the reduction of NBT and resulting in a lower optical density compared to the system without SOD.

To perform the assay, 0.1ml of tissue homogenate was added to 6ml of

reaction mixture containing 0.1M phosphate buffer (pH 7.8), 10mM EDTA, 130mM methionine, 750µM NBT, and 60µM riboflavin. The reaction tubes were exposed to 28W fluorescent light tubes at a distance of 15cm inside a light-proof chamber for 20 minutes. To prevent interference from external light, the tubes were wrapped with black paper both before and after exposure. The absorbance of the reaction mixture was measured at 560nm. The amount of homogenate required to reduce the optical density to half was considered as one unit of SOD enzyme activity.

Estimation of Catalase (CAT): Catalase activity was measured using a modified method as described by Aebi (Aebi, 1984). The assay is based on the degradation of hydrogen peroxide (H_2O_2) by the enzyme. The tissue homogenate was prepared in in 0.66mM phosphate buffer solution. The substrate buffer consisted of 75mM phosphate buffer (pH 7.0) and hydrogen peroxide, which was adjusted to an optical density of 0.45 at 240nm. The change in optical density (Δt) was recorded as the time required for the OD to decrease from 0.45 to 0.4. Catalase activity was then calculated using the following formula: Unit of Catalase Activity = 17 / Δt

Estimation of Protein: Protein content was determined using the method of Lowry et al. (Lowry et al., 1951). The samples were prepared in distilled water at a concentration of 2mg/ml. The protein estimation was carried out in triplicates.

To estimate the protein, 0.5ml of the sample was mixed with 3.5ml of distilled water in a test tube. To this mixture, 5.5ml of Reagent C was added, consisting of 50ml Reagent A (2% Na_2CO_3 in 0.1 N NaOH) and 1ml Reagent B (0.5% $CuSO_4$ in 1% sodium potassium tartrate). The mixture was incubated for 10 minutes. After incubation, 0.5ml of Folin-Ciocalteau phenol reagent was added, and the mixture was incubated for an additional 30 minutes. The absorbance was measured at 660nm against the blank. A standard curve of bovine serum albumin (BSA) was used to determine the protein concentration in the sample, expressed as mg of protein per mg of tissue.

Study of Maximum Lifespan Potential (MLSP): The maximum lifespan potential in Drosophila was evaluated following the method outlined by Bass et al. (Bass et al., 2007). Newly emerged male (n=10) and female (n=10) flies were separated into vials containing their respective culture medium based on the study groups. The flies were transferred to fresh vials with new culture medium every three days. The maximum lifespan potential was recorded as the day on which the last fly in the group died (n=6).

Statistical Analysis

Data are presented as arithmetic mean ± standard deviation. The level of significance between groups was determined using one-way ANOVA, followed by Tukey's post-hoc test for pairwise comparisons.

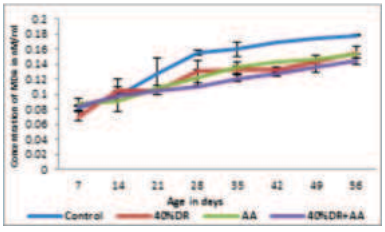
RESULTS

Lipid Peroxidation: Lipid peroxidation was measured in the form of nM of MDA. The results are displayed in Table No.1 and Graph No.1. In all study groups, an age-dependent increase in lipid peroxidation was observed. However, when compared to the control group, lipid peroxidation levels were significantly lower across all experimental groups. Notably, the DR+AA group exhibited a further reduction in lipid peroxidation relative to both the DR and AA groups, with the difference being statistically significant ($p<0.05$). These findings suggest that, DR+AA supplementation exerts a synergistic effect on malondialdehyde (MDA) levels, effectively maintaining lower MDA levels in the DR+AA group. The application of DR+AA supplementation was found to be particularly advantageous, demonstrating a more pronounced beneficial effect.

Table–1 Effect Of Dietary Restriction And Ascorbic Acid Supplementation Separately And Synergistically On The Levels Of Lipid Peroxidation In The Form Of NM Of MDA/mG Tissue In Drosophila Melanogaster

Age of Fly (Days)	Control group	40% diet restricted group (DR) (#)	Ascorbic acid supplemented group (AA)	40% diet restricted + Ascorbic acid supplemented group (DR+AA)
7	0.082± 0.004	0.071± 0.0059***	0.081± 0.003*	0.078± 0.005
14	0.099± 0.022	0.106± 0.0044*	0.092± 0.0001**	0.099± 0.0059*
21	0.127± 0.022	0.106± 0.0054**	0.108± 0.0044*	0.106± 0.0054*
28	0.156± 0.0039	0.131± 0.015**	0.122± 0.0104*	0.11± 0.0001***
35	0.16± 0.01	0.133± 0.0044**	0.136± 0.0077**	0.122± 0.004***
42	0.17± 7.07E5	0.133± 0.004**	0.14± 0.0001***	0.128± 0.007**
49	0.174± 0.0004	0.144± 8.16E5***	0.147± 0.0059**	0.136± 0.008**
56	0.179± 0.0004	0.156± 0.0077*	0.154± 7.07E5**	0.144± 0.009*

Data is represented as arithmetic mean±SD *** p<0.001 versus control (n=6). # Udugade et al. 2016.



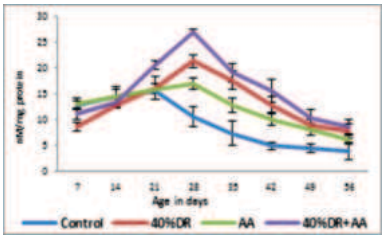
Graph–1 Effect Of Dietary Restriction, Ascorbic Acid Supplementation Separately And Synergistically On The Lipid Peroxidation In D. Melanogaster

Superoxide dismutase: The levels of superoxide dismutase (SOD) enzyme activity are presented in Table 2 and Graph 2. In the control group, SOD activity increased progressively until day 21, after which it declined through to day 56. In contrast, all experimental groups exhibited a steady increase in SOD activity up to day 28, followed by a subsequent decline. Notably, the DR group showed a twofold increase in SOD activity compared to the control group, while the AA group demonstrated a 1.5-fold increase. In the DR+AA group, SOD activity was elevated by 2.5-fold on both day 28 and day 56 relative to the control group ($p<0.001$).

Table–2 Effect Of Dietary Restriction And Ascorbic Acid Supplementation Separately And Synergistically On The Activity Of Superoxide Dismutase In The Form Of Nm/mg Protein In D. Melanogaster

Age of fly (Days)	Control group	40% diet restricted group (DR)	Ascorbic acid supplemented group (AA)	40% diet restricted + Ascorbic acid supplemented group (DR+AA)
7	12.74 ± 0.89	8.61 ± 0.73*	13.12± 0.94	11.05 ± 0.97
14	14.31± 2.13	12.84 ± 0.55	14.33 ± 1.41	13.35± 0.91
21	15.45 ± 1.54	16.16 ± 2.16	15.89 ± 0.99	20.50± 0.87***
28	10.50 ± 1.91	21.12± 1.29***	16.79 ± 1.12***	26.87± 0.56***
35	7.35± 2.43	17.52± 1.81***	12.65 ± 1.38***	19.02 ± 1.85***
42	4.92 ± 0.73	12.87± 1.53***	9.90 ± 1.13***	15.56± 2.29***
49	4.45 ± 0.90	8.93 ± 0.94***	8.06 ± 1.03***	10.41± 1.57***
56	3.87 ± 1.47	7.85± 1.40***	6.19 ± 0.67*	8.59 ± 1.42***

Data is represented as arithmetic mean±SD *** p<0.001 versus control (n=6).



Graph – 2 Effect Of Dietary Restriction, Ascorbic Acid

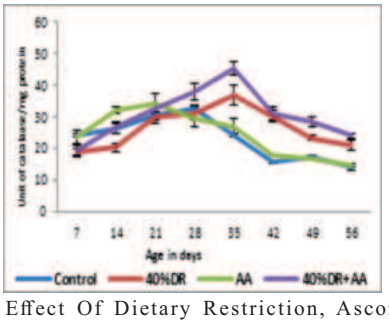
Supplementation Separately And Synergistically On The Levels Of Superoxide Dismutase Activity In D. Melanogaster

Catalase: Catalase activity is shown in Table 3 and Graph 3. In the control group, catalase activity increased up to day 28, whereas in the DR and DR+AA groups, it continued to increase until day 35. In the AA group, catalase activity rose until day 21. Following these periods, a decline in catalase activity was observed, which was age-dependent. A highly significant ($p<0.001$) increase in catalase activity was noted in the DR and DR+AA groups at advanced age compared to the control group. However, no significant increase was observed in the ascorbic acid-supplemented group.

Table–3 Effect Of Dietary Restriction And Ascorbic Acid Supplementation Separately And Synergistically On The Activity Of Catalase Enzyme In D. Melanogaster

Age of fly (Days)	Control group	40% diet restricted group (DR)	Ascorbic acid supplemented group (AA)	40% diet restricted + Ascorbic acid supplemented group (DR+AA)
7	24.16± 0.65	18.61± 1.06***	23.81± 2.05	19.26 ± 1.53***
14	26.51± 1.75	20.40 ± 1.21**	32.01 ± 1.14**	26.56± 1.84
21	29.96± 1.41	30.02 ± 1.88	34.54± 2.72**	32.82 ± 1.62
28	32.76± 0.73	31.46 ± 2.29	29.66 ± 2.75	37.04 ± 2.61 **
35	24.34± 0.48	36.84± 3.20***	27.00 ± 2.42	45.31 ± 2.12***
42	16.04± 0.46	30.69± 1.83***	17.95 ± 0.12	31.32 ± 2.01***
49	17.32± 0.51	23.45± 0.87***	16.74 ± 0.53	28.56 ± 1.57***
56	14.20± 0.93	21.97± 1.40***	14.92 ± 0.41	24.54± 0.82***

Data is represented as arithmetic mean±SD *** $p<0.001$ versus control (n=6).

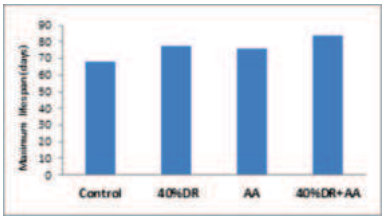


Graph–3 Effect Of Dietary Restriction, Ascorbic Acid Supplementation Separately And Synergistically On The Levels Of Catalase Activity In D. Melanogaster

Maximum life span potential: As shown in Table 4 and Graph 4, the data reveal a significant boost in the maximum lifespan for all experimental groups. The control group had a maximum lifespan of 68 days, while the DR group it was 75.7 days, and the ascorbic acid-supplemented group reached 75.6 days. However, the real breakthrough came with the combined treatment of DR + Ascorbic Acid, where the lifespan soared to an impressive 83.5 days — a remarkable 123% increase! This highlights the powerful synergy between DR and ascorbic acid in promoting longevity.

Table–4 Effect Of Dietary Restriction And Ascorbic Acid Supplementation Separately And Synergistically On Maximum Life Span In D. Melanogaster

Group	Maximum lifespan potential in days
Control group	68 ± 2.20
40% diet restricted group (DR)	77.7 ± 1.67***
Ascorbic acid supplemented group (AA)	75.6 ± 1.41***
40% diet restricted + Ascorbic acid supplemented group (DR+AA)	83.5 ± 2.37**



Graph–4 Effect Of Dietary Restriction, Ascorbic Acid Supplementation Separately And Synergistically On The Maximum Lifespan In D. Melanogaster

DISCUSSION

The results revealed that, the DR group, ascorbic acid supplemented group and DR+ Ascorbic acid cotreated group exhibited a significant reduction in lipid peroxidation levels. Lipid peroxidation, a result of oxidative damage to membrane lipids, is a well-established biomarker of aging (Hornbrook, 1997; Rikans & Hornbrook, 1997; Pratic, 2002; Gallelli et al., 2018; Niki, 2019). Numerous studies have shown a positive correlation between age and lipid peroxidation levels across various animal models and tissues (Massie et al., 1983; Sawada & Carlson, 1987; Spiteller, 2001; Ayala et al., 2014) Similarly, in the present study, an age-related increase in lipid peroxidation was observed in all groups. Dietary restriction is known to mitigate lipid peroxidation (Ward et al., 2005; Wu et al., 2003; Zheng et al., 2005). (Wu et al., 2003; Ward et al., 2005; Zheng et al., 2005). According to Yu (1990), DR reduces oxidative stress by neutralizing free radicals, enhancing the antioxidant system, and promoting the repair of damaged cellular structures (Yu, 1996). DR induces mild stress, which triggers adaptive cellular mechanisms, including increased autophagy (Calabrese & Baldwin, 2002; Xie & Klionsky, 2007). Studies in rodents have demonstrated that a 40% DR regimen reduces oxidative damage, as evidenced by lower levels of thiobarbituric acid-reacting substances (TBARS) and fewer lipofuscin granules compared to ad libitum-fed rats (Rao et al., 1990). Furthermore, DR strengthens organisms against molecular damage associated with aging, particularly in non-mammalian systems (Kaeberlein et al., 2006). Chen & Yu (1994) also found that isolated hepatic mitochondria from DR rats showed reduced mitochondrial oxidant generation and attenuated age-related decline in membrane fluidity (Chen & Yu, 1994).

Age-related deterioration of the antioxidant defense system contributes significantly to increased oxidative stress, leading to further cellular damage. Superoxide dismutase (SOD) plays a crucial role in defending cells by converting superoxide radicals ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). While H_2O_2 can be cytotoxic, it is subsequently broken down into water and oxygen by catalase (CAT) (Mueller et al., 1997; Horst et al., 2006). Several studies have shown reduced activity of antioxidant enzymes such as SOD and CAT with age. For instance, Sohal et al. (1994) reported that 40% caloric restriction increased lifespan by 43% and reduced mitochondrial generation of superoxide radicals and hydrogen peroxide in calorie-restricted mice compared to ad libitum-fed mice (Sohal et al., 1994). In our study, maximum SOD activity was observed on day 28, while CAT activity peaked on day 35 in the dietary restriction (DR) group, suggesting a response to DR. Zabuga et al. (2015) reported similar findings in male Drosophila flies under 50% dietary restriction, where SOD and catalase activity were significantly elevated (Zabuga et al., 2015). Overall, DR effectively reduces oxidative stress and enhances antioxidant defense mechanisms (Weindruch & Sohal, 1997; Meydani et al., 2011).

Supplementation with ascorbic acid (AA) also resulted in reduced lipid peroxidation levels compared to the control group. The increased SOD activity in the AA group highlights the potential benefits of dietary ascorbic acid. However, the increase in CAT activity was not significant in the AA-supplemented group. Ascorbic acid, a water-soluble antioxidant, is well-known for its ability to scavenge free radicals and maintain the redox balance within cells (Sies et al., 1992; Chan, 1993; Beyer, 1994). Supplementing the diet with exogenous antioxidants, whether natural or synthetic, has been shown to enhance the activity of endogenous antioxidants, helping to counteract the damaging effects of stress (Brewer, 2011; Chand et al., 2017). These exogenous antioxidants, including vitamins and polyphenols, work by neutralizing free radicals (Pandey & Rizvi, 2009). In the present study, the group supplemented with ascorbic acid demonstrated a significant

increase in both superoxide dismutase (SOD) levels and catalase activity. Similarly, Khassaf et al. (2003) observed that supplementing with 500 mg/day of ascorbic acid for eight weeks resulted in enhanced activities of SOD and catalase, alongside elevated levels of HSP 60 in human lymphocytes (Khassaf et al., 2003). Furthermore, Ibuki et al. (2020) reported that, ascorbic acid supplementation boosted the activity of key antioxidant enzymes SOD, catalase, and GPx in the salivary glands of streptozotocin (STZ)-induced diabetic rats after 30 days of treatment (Ibuki et al., 2020).

The combined treatment of DR and AA supplementation resulted in significantly lower lipid peroxidation levels. On day 28, the SOD activity in the 40% DR + AA group was the highest among all groups, showing a fourfold increase compared to the control group. Likewise, catalase activity was elevated twofold in the DR + AA group compared to controls. The enhanced SOD activity up to day 28 in all experimental groups, compared to the control, may reflect a maintenance of cellular homeostasis and a delay in the aging process through DR or AA supplementation, or both. The decline in SOD activity after day 21 in the control group could be attributed to genome instability resulting from epigenetic modifications, triggering the aging process, as described by Aguilera and García-Muse (2013) (Aguilera & García-Muse, 2013). In contrast, the AA-supplemented group may have maintained genomic stability for an additional week, as suggested by Brabson et al. (2021), who noted that vitamin C has the potential to reverse epigenetic modifications and regulate gene expression (Brabson et al., 2021). Similarly, DR has been shown to protect against age-related epigenetic changes, as demonstrated by Hahn et al. (2017), who reported that DR mitigates age-associated DNA methylation and induces epigenetic reprogramming in genes related to lipid metabolism (Hahn et al., 2017). In this study, the sustained increase in SOD activity up to day 28 in the 40% DR + AA group suggests a synergistic protective effect of both treatments in maintaining genomic stability and potentially reversing epigenetic modifications, contributing to improved longevity.

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