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Dietary Restriction Initiated from Larval Stage Decreases Maximum Life Span in *Drosophila melanogaster*

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Abstract: *Dietary restriction (DR) is a way of hormesis which stimulates the adaptive response in various animals. This reduces free radical formation and induces turnover of biomolecules by autophagy. In Drosophila melanogaster also we found that, there is decrease in lipid peroxidation and increase in maximum life span when the adult flies are subjected to 20% and 40% DR. In this group DR was initiated after eclosion of flies, i. e. when they become adult. 40% DR was found to be more beneficial in this group. Contradictory results were found when Drosophila melanogaster was subjected to 20% and 40% DR from the larval stage. Lipid peroxidation was found to be at higher level up to 21 day of eclosion which was lowered in later period. Maximum life span was found to be reduced on 20% and 40% DR. The flies subjected to 40% DR from larval stage were found to be more affected.*

Keywords: *Dietary restriction, Adult DR, Larval DR, Lipid peroxidation, Maximum life span potential.*

I. Introduction

Ageing is universal process. Ageing increases the risk of various age related disorders in all species including human being. From many years, gerontologists worked for understanding the causes of ageing and age related disorders like cancer, cardiovascular diseases, diabetes, neurodegerative disorders like Alzheimer's and Parkinson's disease .

Dietary restriction (DR) imposed by dilution of nutrient content without malnutrition [1]. Dietary restriction is one of the remedy to slow down the process of ageing [2]. In 1930's Mac Cay *et al.,* [3] shows the life extension in rat by caloric restriction. DR found to increase lifespan in various organisms from non chordates to primates including yeast, nematode, fruit flies and mammals [4]. In Rhesus monkey, DR shows the positive effects that decreases Blood glucose and insulin level [5] also lowering body temperature [6]. Austad *et al.,* [7] demonstrated the delayed egg laying and reduced fecundity in spider *Frontinella pyramitela.*

Dietary restriction has anti-atherogenic effect that reduces atherosclerosis and oxidative stress in the aorta of apolipoprotein E-deficient mice [8]. The Long-term calorie restriction is highly effective in reducing the risk of developing atherosclerosis in humans [9]. In Pigeon, dietary restriction prevents the age-related accumulation of aortic cholesterol early in life [10]. DR exerts as a mild stress that prevent neuronal loss and cellular damage in Alzeimer's disease (AD) [11] and Parkinson's disease [12].

Roth *et al*., [13] hypothesized that the antiaging effects of caloric restriction is reduced energy expenditure with simultaneous reduction in the production of reactive oxygen species (ROS). ROS refers to free radicals containing oxygen molecules. Free radicals are atoms or molecules containing unpaired electrons in its outermost shell derived from oxidative phosphorylation in mitochondria during cell respiration [14]. They encounter the macromolecules by oxidizing them. Polyunsaturated fatty acids (PUFA) in the cell membrane, oxidation of amino acids in proteins, oxidative oxidation of DNA can be the targets of ROS.

When the amount of free radicals exceeds in the body and decreased body's ability to eliminate or neutralize them, it refers as oxidative stress [15] results in peroxidation products of lipids, usually polyunsaturated fatty acids (PUFAs) leading to aldehyde formation as 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) [16].

II. Material And Method

Animal model:

Fly strain, culture conditions

Wild strain of *Drosophila melanogaster* was obtained from Department of Zoology, Banaras Hindu University Varanasi. They were cultured on maize medium at 21^0C in BOD incubator. Flies were grouped into following groups,

Group I(control)-These flies were grown on maize medium containing maize powder 9gm, crude sugar 8gm, Agar-Agar 3gm, Sodium benzoate 0.66gm and Propionic acid 0.66ml in 200ml drinking water. Lastly, dried yeast spread on the surface of medium.

Group II (Dietary restriction group)-The newly emerged flies were grown on DR maize medium. Sugar and maize powder restricted in this medium.

1. **Larval Diet restriction-**The restriction was done from larval stages. This group was subdivided into:

a. **20% dietary restricted group-**Maize powder and sugar components were restricted by 20%.

b. **40% dietary restricted group-**Maize powder and sugar components were restricted by 40%.

2. **Adult Diet restriction-**The restriction was initiated from the 1st day of emergence. This group was subdivided into:

a. **20% dietary restricted group-**Maize powder and sugar components were restricted by 20%.

b. 40% dietary restricted group-Maize powder and sugar components were restricted by 40%.

Lipid peroxidation

Lipid peroxidation was measured in flies on 7th, 14th, 21st,28th, 35th, 42nd, 49th, 56th day of emergence by the method of Wills [17]. Flies were killed by anesthetic ether. The wings are removed and homogenized at concentration of 2mg/ml in reaction mixture containing 75mM potassium phosphate buffer (pH,7.04), 1mM Ascorbic acid and 1mM $FeCl_3$. Estimation carried out by 0.2ml homogenate and 0.8ml distilled water with 1ml of 20% TCA, 1 ml of 0.67% TBA. This mixture kept in boiling water bath for 10 min. then absorbance was observed at 532nm in spectrophotometer. The values of thiobarbituric acid reactive substance were expressed as nM of malondialdehyde per gram of tissue.

Study of maximum life span potential

For study of maximum lifespan [18], the flies were grouped as group I (control), Group II (dietary restriction groups i.e. larval as well as adult) as mentioned above. The adult flies were transferred on the day of eclosion to the fresh maize medium bottles with 5 males and 10 females allowed to mate for 4 days. For the treatment, newly emerged virgin flies were transferred as per 10 males and 10 females separately on fresh maize medium culture vials. They were transferred to fresh vials every 2 days and observed the day were last fly was dead.

III. Result

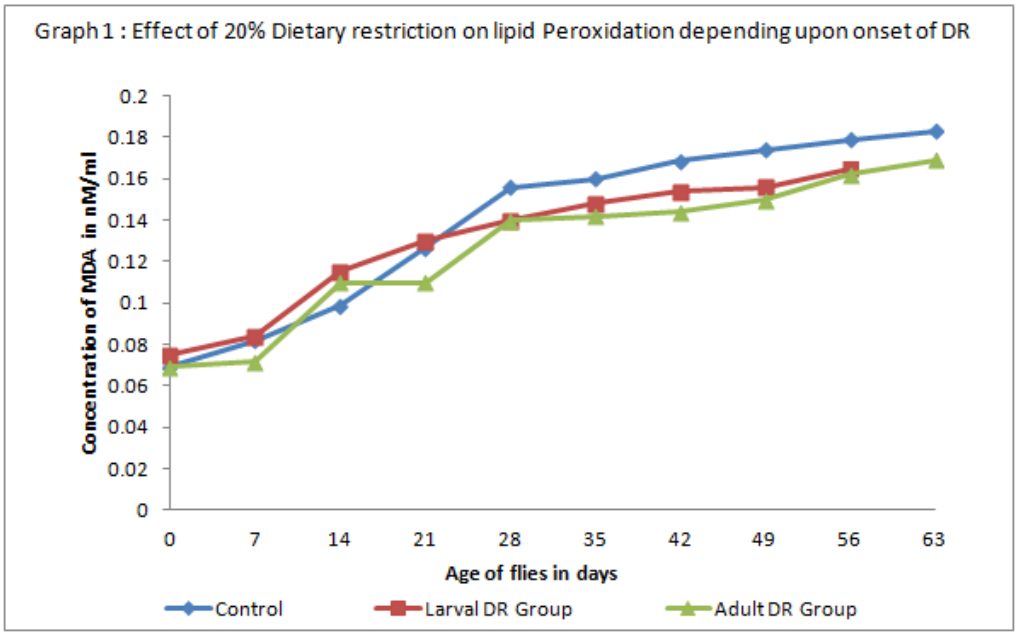
Lipid peroxidation

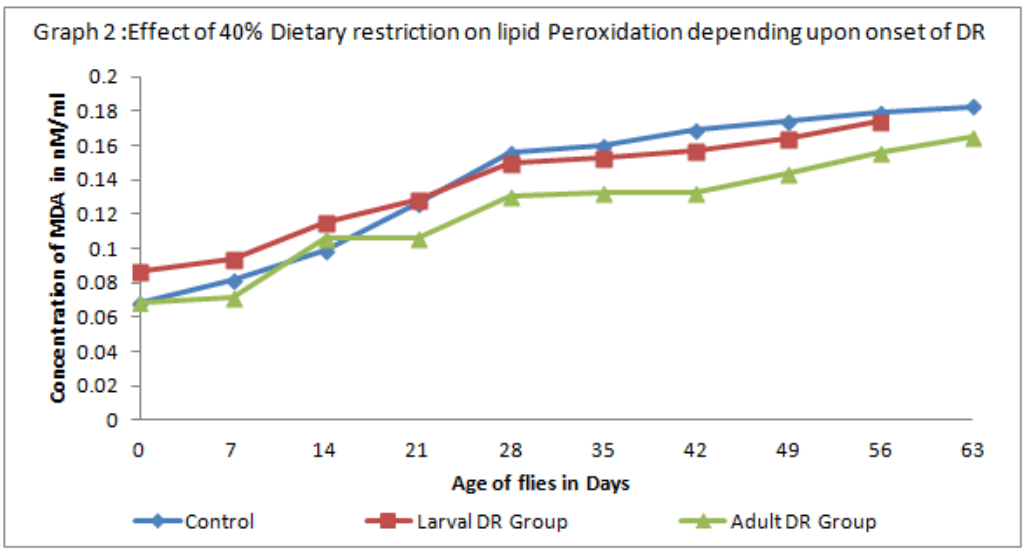
Levels of MDA were found to be increased gradually in all groups with age. In the group where dietary restriction was given from larval stage, the level of MDA was found to be high on the day of emergence as compared to control group. It was higher on day 7, day 14 as well, when compared to control group of same age. On day 21 level of MDA was found to be significantly higher in 20% larval DR group, while non-significantly higher in 40% larval DR group. Then after from day28 the levels of MDA were found to be lower as compared to control in both groups where 20% and 40% diet restricted from larval period. 0.156 nM of MDA was found on day 28 in control group while the same level of MDA was observed on Day 49 in 20% diet restricted group from larval stage. While 0.157nM of MDA level was found on day 42 in 40% diet restricted group. On day 56 there was only 92% lipid peroxidation in 20% larval DR group and 97% lipid peroxidation in 40% larval DR group. (Table 1, Graph 1,2)

In the groups where the diet restriction initiated from adult stage, MDA levels was lower than control group from day 7 onwards. The MDA levels on day 28 in control are same as that on day 56 in 20% DR group while MDA levels on day 42 in control group are higher than the MDA levels on day 63 in 40% DR group. The MDA level on day 63 was 92% in 20% DR group and 90% in 40% DR group as compared to control. When we compare 20% and 40% adult DR groups, the level of MDA was found significantly lower in 40% DR group as compared to 20% DR group and more extended life span in 40% adult DR group. (Table 1, Graph 1,2)

Table 1: Levels of MDA in nM/ml in *Drosophila melanogaster* on Dietary restriction initiated from larval adult stage.

Age of Fly in Days	Control	Larval DR		Adult DR	
		20%	40%	20%	40%
0 Days	0.069±0.015	0.075±0.0074	0.087±0.0074*	0.069±0.015	0.069±0.015
7 Days	0.082±0.004	0.084±0.0051*	0.094±0.0051*	0.0718±0.0059***	0.0718±0.0059***
14 Days	0.099±0.022	0.115±0.015**	0.115±0.015**	0.11±0.0001*	0.106±0.0044*
21 Days	0.127±0.022	0.13±0.0038***	0.129±0.014*	0.11±7.07E5***	0.106±0.0054**
28 Days	0.156±0.0039	0.14±7.07E5**	0.15±0.005*	0.14±5.77E5***	0.131±0.015**
35 Days	0.16±0.01	0.148±0.0108*	0.153±0.0129*	0.142±0.0085***	0.133±0.0044**
42 Days	0.169±7.07E5	0.154±0.0008*	0.157±0.0054*	0.144±5.77E5***	0.133±0.004**
49 Days	0.174±0.0004	0.156±0.0044**	0.164±0.00044*	0.150±0.0059**	0.144±8.16E5***
56 Days	0.179±0.0004	0.165±0.0044*	0.174±0.0072*	0.162±0.0044**	0.156±0.0077*
63 Days	0.183±0.0004	--	--	0.169±0.0001**	0.165±0.0054*





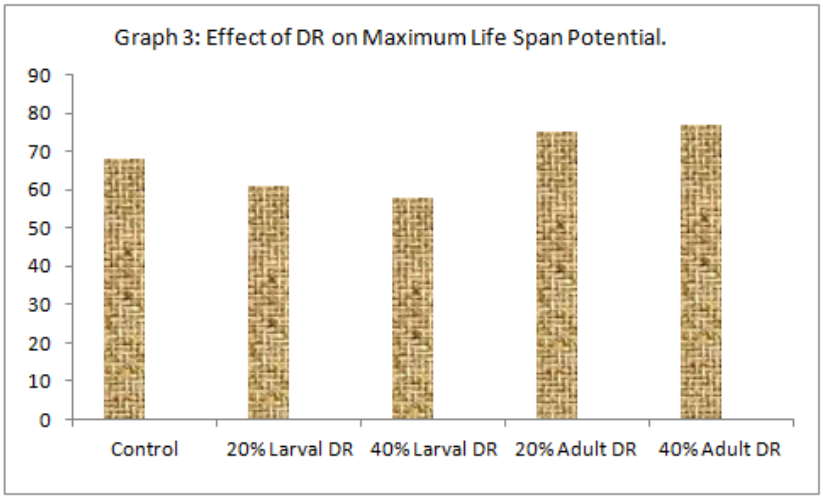
Maximum life span potential

In both groups of larval DR the maximum life span potential was not increased but it was found to be decreased. The maximum life span in 20% larval DR group was 61 days while that of 40% larval DR group was 58 day as compared to 68 days in control group. (Table 2, Graph 3)

In adult DR groups maximum life span potential was found to be increased to 75 day in 20% DR and 77days in 40% DR group. It was 10% and 13% increase in Maximum life span potential in 20% and 40% DR groups respectively. (Table 2, Graph 3)

Table 2: Effect of Dietary restriction on Maximum Life span Potential

Group	Maximum life span (In Days)
Control	68
20% DR form Larval stage	61
40% DR form Larval stage	58
20% DR from Adult Stage	75
40% DR from Adult Stage	77



IV. Discussion

Ageing is gradual deterioration of cellular functions leading to increased chances of death. Harman [19] free radical theory of ageing suggested that, ageing is more or less direct function of the metabolic rate. During normal metabolism also free radicals or more specifically reactive oxygen species (ROS) are generated. They are highly reactive and cause damage to all parts of the cell. These free radicals are scavenged by some enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase, etc. Apart from them some non-enzymatic agents are able to nullify the ROS. Some of the antioxidants are vit.C, vit.E, glutathione etc. Free radicals are essential upto some extend and help in body defense during respiratory burst.

Unscavenged ROS are proved to be deleterious and attack on any biomolecules including lipids, proteins, and nucleic acids. They cause damage to the molecules that they hit. Damaged molecules are handled by lysosomes by the process of autophagy [20,21].

If free radical formation exceeds beyond the limit of scavenger system, repair machinery and lysosomal capacity, the damaged products accumulates in the cell leading to impaired functioning of cells resulted in the cell death. Lipids are the most susceptible biomolecules for free radical attack [22]. Polyunsaturated fatty acid (PUFA) are one of the targets of ROS [23, 24,25]. They are abundantly present in biological membrane [26]. Free radicals hit the PUFA and remove hydrogen atom. By this PUFA become lipid radical. It undergoes rearrangement of intramolecular bonds and reaction with molecular oxygen leads to peroxyl radical generated. Peroxyl radical react with other molecule of PUFA and become lipid hydroxide. Second PUFA molecule turns in lipid radical. Thus the chain of reaction begins. This reaction propagates in ramified form and decomposition of hydro peroxide resulted in alkanes, aldehydes, epoxy and hydroxyl fatty acids, etc. [27].

There are plentiful of evidences showing age wise increased in levels of lipid peroxidation in various experimental animals. In present investigation also we found age related increase in lipid peroxidation in all groups of *Drosophila melanogaster* under study. Lipid peroxidation causes damage to biological membranes. Tsen and Collier [28] demonstrated membrane damage resulted from lipid peroxidation. Lipid peroxidation was found to induce alterations in biological membranes. It causes disturbance of fine structure, loss of functions and permeability of biological membranes [21].

Aim of present study is to develop a way to extend healthy lifespan rather only extend the lifespan. To achieve this purpose, age related damage to the biomolecule has to be reduced. The major damaging agents are the free radicals which should be curtail by all possible means.The simplest possible way to serve this purpose is dietary restriction (DR). Dietary restriction is a reduction in nutrients availability without malnutrition. DR is a mild stress which stimulates adaptive response of the body which is beneficial. Such mild stimulation is termed as hormesis [29].

DR was to found to extend life and delay the onset of age related dysfunctions in various organisms and model systems studied from last 70 years [30, 31] Mair and Dillin [32] showed that, DR modulates the activity of multiple longevity related cellular factors. The increased longevity in the diet restricted group as found in present study may be due to similar mechanism. As there is nutrient depletion mTOR (mechanistic target of rapamycin) activity is reduced. This result is slowing down of cascade of events leads to promote longevity. It also enhances resistance to stress [33,34]. On other hand its stimulates degradation of macromolecules via autophagy, there by reduces accumulated waste and contributes to extension of lifespan [35] which was observed in the group were onset on DR was from adult stage.

Reduced level of lipid peroxidation in all diet restricted groups in this investigation may be results due to decreased oxidative stress as well as increased autophagy. Dietary restriction is a diverse and effective

modulator of oxidative stress [36]. Kim et al., [37] showed that, long term DR and chronic exercise reduced free radical formation decreased free radicals may be scavenged by antioxidant system of the cell. Secondarily, lower number of free radicals causes less damage to cellular components which can be repaired by the repair system; non-repairable molecules are subjected to autophagy by lysosomes and degraded for cellular turn over. Thus, DR has duel effect as it reduces free radicals and damage caused by them on one hand while enhances autophagy and prevent accumulation of damaged macromolecules in other hand. Both this phenomenon may be responsible for reduced level of lipid peroxidation in diet restricted groups.

Higher levels of MDA on day one of emergence in the group were DR was started from larval stage indicates increased stress during metamorphosis. The larval stage is the period of development where a larva has to synthesize and store high amount of proteins and other materials for the process of metamorphosis. So during larval stage there is very higher demand of energy. Restriction of diet during this stage may interfere with this purpose and leads to malnutrition rather than hormesis. This stress and energy constrain reflex in the form of higher level of MDA during early age of adult life and so also on the maximum lifespan. DR in adult stage of this group help it to overcome the stress which results in lower level of MDA from day 28 onwards but the long term effects was not beneficial resulted in shorter lifespan in both the groups of larval DR. It was shows that, the beneficial effects of DR are pronounced when it initiates in early age [38]. Our study indicates that onset of DR after completing developmental stages and achieving the adult stage is beneficial, otherwise it may prove hazardous.

DR from adult stage that is after emergence of flies was found to be beneficial by all means. It shows lower level of MDA as well as extended lifespan in both 20% and 40% DR groups. Low levels of MDA indicate lower damage to lipids higher repair and higher recycling by autophagy. This can be considered as signs of healthier conditions as compared to control group flies. This reflects in extended lifespan. The results showed that 40% adult diet restriction is more beneficial than 20% DR. In 40% DR group hormesis level is higher than 20% DR. This might be stimulating the adaptive response strongly in this group, which reflects in the results.

V. Conclusion

In conclusion we can say that DR is one of the easier ways to have healthy and long life as shown in *Drosophila melanogaster*. Onset of DR during developing period is not beneficial rather harmful. DR in adult stage is very effective and beneficial.

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